

Paper of aflatoxin with nanis essay



The active antifungal substance(s) were separated, semi purified and characterized as a protein substance. The semi purified substance inhibited spores formation, thinned the conidiophores and deformed both strigmata and conidial heads. Key words: *Aspergillus niger*, *Streptomyces noursei*, ochratoxin A. INTRODUCTION Growing mould may produce toxic secondary metabolites, such as mycotoxins. Among hundreds of fungal secondary metabolites are mycotoxins which include aflatoxins (AFL), deoxynivalenol, fumonisins (FB), ochratoxin A (OTA), and zearalenone.

They are of major health concern for humans and domestic animals (Miller, 1994). Mycotoxins can enter into the human food chain directly through foods of plant origin and indirectly through foods of animal origin (Kovacs, 2004). Many types of food products in the markets have been reported to be contaminated with AFL or metabolites of AFL. These include tree nuts, peanuts, figs, melon seed, pumpkin seed, sesame seed, sunflower seed, lotus seed, coix seed, red pepper, white pepper, nutmeg, paprika, mixed spices, rice, corn, mixed cereals, chilies, and copra (Wilson, 2002).

OTA has been found in many cereals, raisins, cocoa products, green coffee beans, wine, soybeans, grape juice, pork sausages (Pittet, 2001), spices, and herbs (Patel et al. , 1996). Ueno et al. (1991) were the first to report on ochratoxin A production by a black *Aspergillus* species, *Aspergillus foetidus*. This was later confirmed by Teren et al. (1996) and Magnoli et al. (2003). Abarca et al. (1997) reported that two strains of *Aspergillus niger* produced OTA, and confirmed in numerous studies (Abarca et al. , 2003; Suarez-Quiroz et al. 2004). Horie (1995) reported OTA in *Aspergillus carbonarius*, and confirmed by Heenan et al. (1998), Varga et al. (2000), Abarca et al. (2003)

and Sage et al. (2004). Biological control means the use of living agents to control pests or plant pathogens and offers an important alternative to synthetic chemicals. The use of bacteria like *Pseudomonas* sp. and *Bacillus* sp. , have been investigated due to their properties to produce antifungal metabolites and protect plants from fungal infection (Siddiqui et al. , 2005; Nourozian et al. , 2006).

Biological control is being increasingly considered by the scientific community as a reliable alternative to pesticide utilization in field and in post-harvest. This biological approach is highly desirable for controlling fungal growth on grapes, helping to reduce the amount of *Corresponding author. E-mail: com Allam et al. 667 agrichemical residues in grapes, wine and related products (Cabras and Angioni, 2000).

Actinomycetes are antibiotic producers that are capable of generating 75% of all known antibiotic products. Actinomycetes are a group of filamentous, Gram-positive bacteria (Williams et al. 1993). These organisms are aerobic, saprophytic, and mesophilic forms whose natural habitat is the soil. Several members of the Actinomycetes are known to be producers of important secondary metabolites, including antibiotics, herbicides, and growthpromoting substances (Connell, 2001). Over 55% of antibiotics have been isolated from the genus *Streptomyces* (Embley, 1994), and more than 400 species of the genus *Streptomyces* are mostly soil saprophytes. The gram-positive bacterium *S. noursei* ATCC 11455 produces a complex mixture of polyene macrolides generally termed nystatins (antifungal agent).

The main objective of this study is to prevent or inhibit the growth of ochratoxin producing *A. niger* using biological method. In this connection, the <https://assignbuster.com/paper-of-aflatoxin-with-nanis-essay/>

potential effect of Actinomycete antagonistic to the test organism was focused. MATERIALS AND METHODS Source of used bacteria True and lactic acid bacteria (LAB) used in this study were obtained from Microbiology unit, Bacteriology Laboratory, Faculty of Science, Tanta University. The used Actinomycetes were isolated from soil samples collected from different localities in Egypt, namely Tanta, El-Mansoura, Kafr El-Zayat and El-Mehala El-Kobra.

Soil samples were taken after the removal of about 5 cm of the surface, and then kept in clean plastic bags. Over the surface of solidified starch nitrate agar plates, 0.2 ml samples of the soil dilution were spread out with a sterilized glass rod. The plates were then incubated at $28 \pm 2^\circ\text{C}$ for 7 days. Biological control Different bacterial and Actinomycete isolates were grown in their corresponding suitable liquid media (Nutrient Broth for true bacteria, de Man, Rogosa and Sharpe (MRS) for LAB and starch nitrate for Actinomycetes) for 24 h and 7 days, respectively.

Aliquots (100 μl) of each cell free extract which was previously extracted by milling the cells in sterile saline were applied on the holes, and agar diffusion method was applied. After incubation for 5 days, the diameters of inhibition zones were measured. Characterizations of Actinomycete isolate number 3 For complete identification of the most fungal and ochratoxin antagonistic isolate, several physical, morphological and biochemical properties were examined.

The criteria described in keys of identification (Kuster, 1972; Nonomura, 1974; Szabo et al. , 1975; Szabo and Csontos, 1975) were followed. Bergey's

Manual of Systematic Bacteriology (Williams et al. , 1989) and Bergey's Manual of Systematic Bacteriology (Holt et al. , 1994) were followed for identification. These fall into the following classes: Microscopic characterization Light and scanning electron microscopes in Electron Microscope Unit, Tanta University (model JEOL, JSM-5200 LV) were used for the characterization of Actinomycetes.

Determination of diaminopimelic acid (DAP)-isomer Diaminopimelic acid isomer was determined according to the method of Becker (1964) after modifications as follows: The Actinomycete isolate 3 was grown under shake culture conditions at 28°C in nutrient yeast extract broth and the cells were collected after the achievement of a maximal growth 1 mg of the dried bacterial cells were hydrolyzed with 1 ml 6 N HCl in a sealed Pyrex tube held at 100°C for 18 h. After cooling, the sample was filtered through Whatman no. 1 filter paper, which was then washed with 1 ml water.

The filtrate was dried two or three consecutive times on a rotary evaporator under reduced pressure at 40°C to remove most of the HCl. The residue was taken up in 0.3 ml water and a volume of 5 μ l was then spotted on a thin layer macrocrystalline cellulose plate (Art 5577 Dc- Plastikfolien cellulose 20 \times 20 cm, layer thickness 0.1 mm. Merck). For separation of amino acids, the following solvent mixture was used: Methanol-water- 10 N HCl – Pyridine (80: 17.5: 2.5: 10, by volume). Amino acids were detected by spraying with acetic ninhydrin (0.1, w/v), followed by heating for 2 min at 100°C.

Diaminopimelic acid (DAP) spots were olive- green fading to yellow, whereas the other amine acids exhibited purple spots. Physical and nutritional factors

influencing the growth and antifungal activity of *Streptomyces* sp. To find out the best growth and antifungal activity of the best antagonistic organism, we study the effect of different media, pH, temperature, carbon and nitrogen sources. Partial purification of antifungal substance(s) produced by experimental Actinomycete isolate Fractionation by salting out with ammonium sulfate A modified technique of Jakoby (1971) was used.

Different concentrations of ammonium sulfate (25, 50, 75 and 90%, (w/v)) were investigated. The supernatant – ammonium sulfate mixture was kept for 30 min at 4°C before being separated by centrifugation. The precipitate was dialyzed in a dialysis bag in buffer in a refrigerator at 4°C over night until the protein precipitate inside the bag became free from excess sulfate.

Protein analysis Native electrophoresis The methods described by

Stegemann et al. (1985) were applied as follow: Gel electrophoresis:

Dissociating polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to a protocol proposed by Laemmli (1970).

Subunit molecular weight estimation by SDS-PAGE: The method of Weber and Osborne (1969) was used to determine the apparent (subunit) molecular weight of proteins dissolved or extracted in the presence of sodium dodecyl sulfate (SDS). Mode of action of antifungal substance: Different concentrations (0. 10, 0. 25 and 0. 50%) of the active protein extracted from 668 Afr. J. Biotechnol. Table 1. Antifungal activity of cell free extract of different microorganisms against *A. niger*.

Isolate number Actinomycetes 1 2 3 4 5 6 7 True bacteria 8 9 10 Bacillus pumilus Lactic acid bacteria 11 12 13 Lactobacillus plantarum Lactobacillus

acidophilus Lactobacillus bulgaricus Diameter of inhibition zone (mm) 25. 00
20. 00 33. 00 0. 00 0. 00 16. 00 0. 00 bacterial isolates were represented by
4 isolates of true bacteria, 6 isolates of lactic acid bacteria and 7 isolates of
Actinomycete are shown in Table 1. Among different bacteria, isolate 3 of
Actinomycete (Photo 1) was the best bacterial isolate that revealed the
highest antifungal activity against *A. niger*.

Factors influencing growth and antifungal activity of isolate 3 Figures 1, 2, 3,
4 and 5 illustrate the effect of different conditions on growth of isolate no 3.
The optimum growth was on starch nitrate medium containing starch as
carbon source and potassium nitrate as nitrogen source, at 30°C, pH 7.
Characterization and identification of Actinomycete isolate no 3 Out of the 7
isolates of Actinomycetes, organism no 3 was the most active organism that
inhibited the growth of ochratoxin A producing *A. niger*. It was subjected to
further studies in order to be characterized and identified.

Light microscopic examination showed the shape of the branches arranged
in whorls and spiral spore chain (Photo 2). Scanning electron microscope
(Photo 3) showed the spiny spore surface. The data obtained from the
previous characterization program (Table 2) suggesting that isolate no 3
belong to genus *Streptomyces* as indicated by the colour of its aerial mycelia
and the presence of LL-DAP in the cell wall, spore form, physiological and
biochemical characteristics. On the basis of the pervious characters, this
isolate showed to be similar to *S. noursei*.

Thus, it could be concluded that the Actinomycete isolate no 3 is suggestive
of being related to *S. noursei* and thus could be given the name *S. noursei*.

Extraction and semi-purification of the antifungal substance Here, a study on the purification of antifungal substance extracted from the culture broth of the tested *S. noursei* was carried out. Fractionation by salting out with ammonium sulphate Ammonium sulphate was the best precipitant agent used to separate the antifungal substance from *S. noursei* and showed the highest antifungal activity. Characterization and antifungal substance semi-purification of the . 00 0. 00 0. 00 15. 00 0. 00 23 0. 00 28 15 25

Actinomycete isolate 3 were incubated with *A. niger* on Czapek's Dox media. Changes in morphology of fungi were photographed under Scanning electron microscope (model JEOL, JSM-5200 LV) in Electron Microscope Unit, Tanta University. Statistical analysis One-way analysis of variance (ANOVA) was carried out. Separation between different means was carried out according to Duncan multiple range test (LSR) and the simple linear correlation analysis (r) were carried out according to SAS (1985) software for windows version (6. 2). All experiments and analytical determinations were replicated at least three times. RESULTS Control by antagonistic microorganisms Effect of different bacteria on ochratoxin producing *A. niger* the growth of In this experiment, different types of bacteria were tested to control the growth of the common fungal producer of ochratoxin (*A. niger*) which was previously isolated and identified from herbs and medicinal plants in Egypt. The well diffusion method was used to determine the antifungal activity of different bacterial isolate.

The different The antifungal substance was dialyzed by dialysis bag Allam et al. 669 Photo 1. Antifungal activity of the selected Actinomycete isolate 3 on the growth of the common producer of ochratoxin (*A. niger*), (a) control, <https://assignbuster.com/paper-of-aflatoxin-with-nanis-essay/>

(b) supernatant of isolate 3. to remove the excess of ammonium sulphate.

Gel electrophoresis of protein polyacrylamide gel and SDS-PAGE The

obtained antifungal substance was by native performed to native

polyacrylamide gel and SDS-PAGE to illustrate the subunits structure to confirm the degree of purity.

In the case of native polyacrylamide gel, it was presented as one thick band (Photo 4). The final eluted proteins were subjected to SDS-PAGE according to the method of Laemmli (1970). Four bands were observed in the sample on SDS-PAGE (Photo 5). The molecular weights 670 Afr. J. Biotechnol. 35 30 25 20 15 10 5 Diameter of inhibition zone (mm) Dry weight (g/L) 0 Figure 1.

Effect of different media on the growth and antifungal activity of isolate no 3. 60 50 40 30 Dry weight (g/L) 20 10 0 5 10 15 20 25 30 35 40

Diameter of inhibition zone (mm) Temperature °C Figure 2. Effect of different temperatures on the growth and antifungal activity of isolate no 3. of the protein subunits were approximately 12, 35, 41 and 150 kDa. It means that the active substance is a group of four compounds, collected with each other to give their antifungal activity. Mode of action of the antifungal substance Different concentrations of the antifungal substance were mixed with the growth media of fungus *A. niger* to give the concentrations; 0, 10, 0.5 and 0.50%, and incubated for 5 days to show the mode of action of this substance on the growth of *A. niger*.

The result (Photo 6, 7 and 8) illustrated the inhibition of spores formation, thinning of conidiophores and deformation of both strigmata and conidia by increasing the concentration of the semi purified antifungal substance

produced by *S. noursei*. DISCUSSION This study aimed to control contamination of ochratoxin A Allam et al. 671 60. 00 50. 00 40. 00 Dry weight (g/L) Dry weight (g/l) 30. 00 20. 00 10. 00 . 00 Diameter of inhibition zone (mm) 3 4 5 6 7 8 9 pH Figure 3. Effect of different pH values on the growth and antifungal activity of isolate 3. 35 30 25 20 15 10 5 Diameter of inhibition zone (mm) Dry weight (g/L) 0 Figure 4. Effect of different carbon sources on the growth and antifungal activity of isolate 3. 35 30 25 20 15 10 5 0 Diameter of inhibition zone (mm) Dry weight (g/L) Figure 5. Effect of different nitrogen sources on the growth and antifungal activity of isolate 3. 672 Afr. J. Biotechnol. Photo 2.

Light micrograph of isolate (3) grown on different starch nitrate media for 7 days at 30°C at magnifications; (a) x40, (b) x25 and (c) x100. producing *A. niger* in common medicinal and herbal plants that used in Egypt by using biocontrol organism. Allam et al. (2008) detected the fungal contamination of 22 investigated samples of herbal and medicinal plants in Egypt. A total of 7 species of fungi belonged to 5 genera were isolated and identified as *Aspergillus*, *Penicillium*, *Fusarium*, *Botrytis* and *Cladosporium*. The greater number of species was related to the genus *Aspergillus*, including *A. iger*, *A. flavus* and *A. terreus* and the formers were the most dominant and the responsible one for ochratoxin A production was *A. niger*. In the present study, biological control of ochratoxin A producing *A. niger* using antagonist bacteria and Actinomycetes was applied through screening program using liquid cultures. The program showed that Actinomycete isolate no 3 was the most potent organism which gave the best antifungal activity. Subsequently, it was subjected to further studies in order to be characterized and identified.

It was found that the selected isolate can grow well on starch nitrate medium, it is aerobic, spore forming, Gram positive, isolated from soil, spore mass was gray. The aerial mycelium with long straight filaments bearing at more or less regular spindles, the vegetative mycelium produced branched mycelium with the presence of LL-DAP in cell wall. All of the previous characters confirmed that the selected isolate belong to the genus *Streptomyces*. Microscopic examination showed spiny spore surface and spiral spore chain.

The growth on carbon and nitrogen source in synthetic media and other characters are useful in species determination. Following the international keys of Kuster (1972), Nonomura (1974), Szabo et al. (1975) and Szabo and Csotros (1975), the survey of literatures on the description of *Streptomyces* species was done with these keys. In addition, Williams et al. (1989) and Holt et al. (1994) indicated that this isolate belongs to *S. noursei* and thus the name *S. noursei* is given. The polyene macrolide antibiotic nystatin produced by *S. noursei* is an important antifungal agent. The nystatin molecule contains a polyketide moiety represented by a 38-membered macrolactone ring to which the deoxysugar Allam et al. 673 Photo 3. Photography of scanning electron microscope showing the spore surface of isolate (3) at magnifications; (a) x7500 (b) x20000. mycosamine is attached. Molecular cloning and characterization of the genes governing the nystatin biosynthesis is of considerable interest because this information can be used for the generation of new antifungal antibiotics (Brautaset et al. 2000).

The present results showed the promotion or inhibition of biological control organism to inhibit the fungal growth as well as the presence of ochratoxin

on the contaminated herbs may be related to the different chemical contents which are present in each type of herbs. This hypothesis led to an examination of the effect of different factors which may be present in the surround environment of medicinal and food herbs on the growth of *S. noursei* and its antifungal activity. Fractional precipitation of crude antifungal substance produced by *S. noursei* was performed by using different concentrations of ammonium sulphate. Results obtained showed that the ammonium sulphate at concentration 50% was the most suitable to give the highest antifungal activity. The use of ammonium sulphate for protein extraction was reported by Chitte and Dey (2000) on *Streptomyces megasporus*, Patcharaporn et al. (2008) on *Shizophyllum commune* BL23, Cheng et al. (2006) on *B. subtilis* DC33 and Wang et al. (2007) on *B. subtilis* LD-8547. 674 Afr. J. Biotechnol. Table 2. Characterization and identification of isolate (3).

Characteristic Morphological characteristic
Characterized Aerial mycelium
Branching Substrate (vegetative) mycelium Spore mass Spore surface Gram
reaction Motility pH Temperature Cell wall hydrolysis: Diaminopimelic acid
(DAP) Physiological and biochemical characteristic Production of melanin on:
Peptone- yeast extract iron agar Tyrosine agar medium Tryptone yeast
extract broth Nitrate reduction H₂S production Hydrolysis of: Starch Protein
Lipid Cellulose Casein Pectin Degradation of: Xanthin Esculin Tyrosine
Utilization of carbon sources: D-Xylose D- Glucose D- Galactose Sucrose L-
Rhamnose Mannitol L- Arabinose Raffinose Meso-Inositol D- Fructose

Isolate 3 Cottony appearance The aerial mycelium with long straight filaments bearing at more or less regular spindles. Branching arranged in

whorls. Branches of verticillate are produced at its apex spiral spore chain. 0.9 – 1.2 μ m in diameter, the vegetative mycelium produced branched mycelium Gray Spore surface are spiny + Non Motile 6. 5-8 Range between 26-32°C LL-DAP + + + - + + + + + + Allam et al. 675 Table 2. Continue. Utilization of nitrogen sources: L- Asparagine L- Tryptophane L- Glutamic acid Antibiosis *Aspergillus niger* *Bacillus subtilis* *Candida albicans* + - + + Photo 4. Photograph to confirm the purified protein on native polyacrylamide gel. Photo 5. SDS-PAGE photograph of the purified protein. M, molecular weight marker; 1, purified protein.

The results herein indicated that native polyacrylamide gel give one thick band but the active substance composed of four proteinaceous compounds to give its activity against *A. niger*. The molecular masses of these compounds were estimated to be 12, 35, 41 and 150 kDa respectively on SDS-polyacrylamide gel electrophoresis. The semi purified substance showed inhibition of spore formation, thinning of conidiophore and deformation of both strigmata and conidial head. By increasing the concentration of the protein substance, the changes and deformation of fungus increased. This implies that the extracted protein substance from *S. noursei* makes desporulation and deformation to *A. niger*.

This is in agreement with Taechowisan et al. (2005). Generally using *S. noursei* as biological control against (*A. niger*) could be considered as one of the most important methods for controlling the growth and ochratoxin A production. In addition the antifungal substance extracted from *S. noursei* showed inhibition Photo 6. Photography of Scanning Electron Microscope showing the common producer organism for ochratoxin (*A. niger*) at

magnifications, x 750. 676 Afr. J. Biotechnol. Photo 7. Photography of Scanning Electron Microscope reveals the effect of antifungal substance at dose (0. 25%) on *A. niger* at magnifications, x 750. Photo 8. Photography of Scanning Electron Microscope reveals the effect of antifungal substance at dose (0. 50%) on *A. niger* at magnifications, x 750. of spore formation, thinning of conidiophore and deformation of both strigmata and conidial head.