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RESEARCH ARTICLE

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Purification and Characterization of Antifungal Protein from Bacteria

Fadia Falah Hassan[1]
[1]Department of Biology, College of Education for Pure science / Ibn-Alhaithum, University of Baghdad, Baghdad, Iraq
Email: fadia.f.h@ihcoedu.uobaghdad.edu.iq

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ABSTRACT

Fungal infections have dramatically increased over the past few years, as evidenced by an increase in the number of deaths from fungal infections including AIDS, cancer, etc. New antifungal treatments must be developed to meet the global challenge of preventing fungal infection and mycotoxin contamination of food and feed. Due to their characteristics like diversity and function, antifungal spectrum, mechanism of action, high stability, and the availability of biotechnological production methods, antimicrobial peptides and proteins (AMPs) with antifungal activity are attracting significant attention as natural antifungal agents. This study focused on the isolation of antifungal protein-producing microorganisms by using a crowded plate technique from the soil along with the purification of antifungal protein by methods like ammonium sulfate precipitation, solvent extraction, etc. along with this characterization of antimicrobial protein was carried out by polyacrylamide gel electrophoresis. Antifungal potential of purified protein was tested using the agar well diffusion method against respective pathogenic fungi**.** Bacillus species having good antifungal activity have been isolated from the soil. Efficient extraction of antifungal compound produced by isolated Bacillus species has been done using ammonium sulfate precipitation as well as a solvent extraction method. The purified antifungal protein exhibits good antifungal activity against food-borne pathogens like Aspergillus niger and Candida albicance. Therefore, the isolated Bacillus strain and its potent antifungal protein could be used in various food and biomedical applications.

Keywords: Fungi, Bacteria, Antifungal Protein, Purification, Characterization.

INTRODUCTION

Fungi are a type of microorganism that can be extremely harmful to agricultural crops, farm animals, and humans. [1] As a result, several researchers have focused their time and attention to the hunt for organic chemicals capable of combating fungal infection. In addition to tiny chemical molecules with antifungal action, antifungal proteins have received attention. [2] Antifungal proteins are now known to be produced by a wide range of animals, including humans, other vertebrates, invertebrates, plants, fungi, and bacteria. [3] There is a diverse range of antifungal proteins with various architectures. Other antifungal proteins besides the well-known glucanases, chitinases, thaumatin-like proteins, defensins, and ribosome-inactivating proteins include lipid transfer proteins and protease inhibitors. So we undertook the present investigation for the isolation of antifungal protein-producing microorganisms. [4,5,6]. The majority of the microorganisms that produce antimicrobial agents are found in soil. Numerous different soil bacteria and fungi can be inhibited by an antibiotic produced by a microbe. Since many years ago, Bacillus species have been utilized in the production of industrially important

enzymes, antibiotics, and pesticides. [7] . Numerous crops have demonstrated the effectiveness of biocontrol agents. Numerous studies have been conducted on biocontrol agents using naturally occurring antagonistic microbes. Because of their advantages over Gram-positive bacterial and fungal biological control agents, many strains of the genus Bacillus have attracted a lot of attention as biological control agents.[8]

MATERIALS AND METHODS

Screening of Antifungal Producers:

The isolation of antifungal producers was carried out by a crowded plate technique [9] of the soil from some area of Baghdad city. In that one gram of soil sample was accurately weighed and transferred to 10 mL of sterile water, mix well, and then placed on a rotary shaker at 120 rpm for 15 min for proper mixing of soil in water. Then the serial dilution of the soil sample was carried out as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} and spread on Nutrient agar plates. The plates were incubated at room temperature for 24 hrs. After 24 hrs incubation soft molten agar of MGYP agar((Malt Extract 0.3%, Glucose-1.0%Yeast extract-0.3% Peptone-0.5% Agar 1.5%, Distilled water 100 ml, pH adjusted to 4.0).) medium containing spore of Aspergillus niger and C.albicabns was overlay on plates. Then the plates were further incubated at room temperature for 48 to 72 hrs and observed for colonies inhibiting fungal growth. The colonies showing inhibition were isolated and studied for their Gram nature further biochemical tests were carried out for the confirmation of isolation up to the species level. [10]

Production of Antifungal Compound:

The production of the antifungal compound was carried out from isolated organisms. For this purpose, initially, the spore suspension of A. niger and C.albicans has inoculated in 50 ml MGYP broth and incubated on a rotary shaker at room temperature. [11] After 24 hrs of incubation, mycelia were collected and added into the 100 ml Muller and Hinton broth. Then the medium was sterilized and inoculated with an isolated organism (Iso-A). The incubation was carried out on a rotary shaker at room temperature. After 24 hrs incubation, a 10 ml aliquot was taken and centrifuged at 8000 rpm for 10 min. The collected supernatant was subjected to an antifungal assay against fungal pathogens. [12]

Extraction of Antifungal compound:

a) By ammonium sulfate precipitation:

After production of the antifungal compound broth was centrifuged at 8000 rpm for 10 min at 4˚C and supernatant was collected. Then the collected supernatant was subjected to ammonium sulfate precipitation at different saturation such as 40%, 50%, 60%, and 70%. [13] The flask was kept in the refrigerator at 4˚C overnight and the precipitate was collected by centrifugation at 8000rpm for 10min at 4˚C. The precipitate was dissolved in 50mM phosphate buffer having pH-7.0 and dialysis was carried out in the same buffer overnight. Then this was subjected to check antifungal activity.[14]

b) Extraction by organic solvent:

The supernatant collected after the production of antifungal compounds was distributed in two equal parts. One part was added with an equal volume of chilled ethyl acetate and the other part was added with an equal volume of chilled chloroform. [15] Then the mixtures were kept in a separating funnel and shaken thoroughly. Afterward, the mixture was kept for clear separation of two layers and the organic solvent layer was separated. The collected organic solvent was concentrated up to 2ml and used to check antifungal activity against the test fungal pathogen.[16]

Antifungal assay:

The culture of Aspergillus niger and Candida albicans , was collected from NCIM (National Collection of Industrial Microorganisms), India. All cultures were transferred and maintained on a Glucose Yeast extract Malt extract medium. The antifungal activity of the sample was checked by the agar diffusion method [17]; in that, the MGYP [HI-Media] plates were spread with fungal spore suspension and wells were prepared with the help of sterile cork borer. The prepared wells were added with extracted supernatant and kept for diffusion in cold conditions for 10 min. Then plates were incubated at room temperature and plates were observed for inhibition of the growth of fungal mycelia around the well.[18] Here ketoconazole is used as positive control and ethyl acetate as a negative control.

SDS-PAGE analysis:

After ammonium sulfate precipitation the fractions showing antifungal activity were subjected to SDS-PAGE analysis. The 12% resolving gel and 4% stacking gel were used with molecular weight markers for the determination of molecular weight.[19].

RESULTS AND DISCUSSION

Isolation of antifungal producers:

The soil sample spread on nutrient agar plates with overlaying of PDA containing fungal spores showing after 48 hr few of the colonies showing mycelia inhibition. The two colonies were selected as potent producers and named Iso-A. The Iso-A was Gram-positive in Gram nature and showed rod-shaped morphology under microscopic observation. [Fig.2] Biochemical tests confirmed that the isolated microorganism is belonging to the Bacillus species. [10] [Table 2]

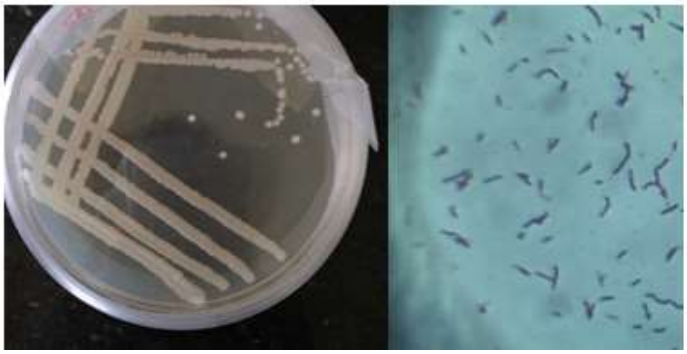

FIGURE.1: four quadrant streaking and Gram staining of isolated organism.

TABLE 1: colony Characteristics of well-isolated isolate

Size	**Shape**	**Color**	**Margin**	**Surface**	**Elevation**	**Opacity**	**Consistency**
2 mm	Circular	Off white	Erose	Rough	Convex	Opaque	Sticky
Gram nature		**Motility**					
Gram-positive		Non motile					

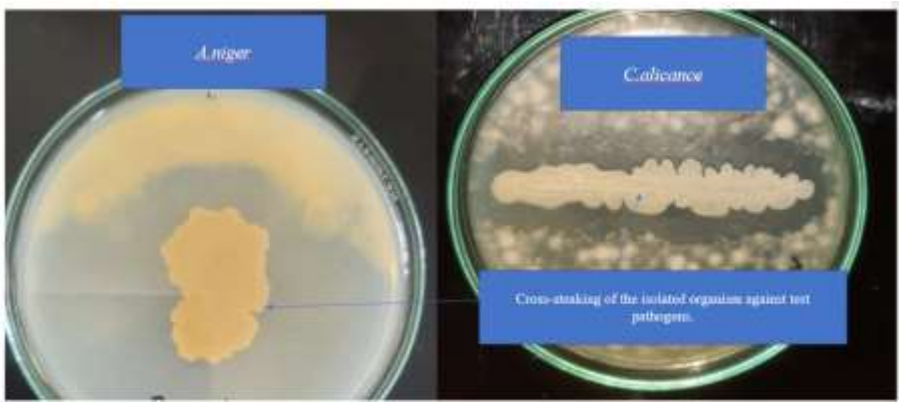

FIGURE.2: cross streaking of the isolated organism against test pathogen.

TABLE 2. Biochemical tests of isolated organism.

Sr. No	Biochemical tests	Result
1	Gram staining	Gram-positive
2	Casein hydrolysis	positive
3	Starch hydrolysis	Positive
4	Nitrate reduction	Negative
5	Methyl red	Negative

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6	Indole production	Negative
7	Citrate utilization	Negative
8	Voges-Proskauer	Negative
9	Catalase	Positive
10	Urea	Positive
11	Oxidase utilization	Positive

Production and Extraction of antifungal activity of partially purified antifungal compound:

Potent organisms were found to produce the antifungal compound in presence of broth containing autoclaved mycelia and candida cells.The antifungal activity was found to be more against A. niger than the c.albicance.

The antifungal compound produced in broth extracted by organic solvent has not shown any activity against A.niger but shown activity against C.albicans which indicates that the compound is not soluble in an organic solvent. The compound was precipitated by ammonium sulfate at 70% saturation have shown more antifungal activity than organic solvent extraction method (Fig.3 and Table.2)

FIGURE.3: Antifungal activity of the partially purified [Ammonium sulfate and ethyl acetae extraction] antifungal compound

TABLE 3. Zone of inhibition of partially purified antifungal protein against the test pathogens.

Sr. No.	Test organisms	Zone of inhibition in mm			
		Ammonium. Sulphate	Solvent extraction	Positive control	Negative control
1	A.niger	22	12	25	-
2	C.albicance	18	12	20	-

SDS-PAGE analysis

As the antifungal compound produced by isolate precipitated by ammonium susulfatehich confirms its nature as a protein. So the extracted protein was analyzed by SDS-PAGE. It was found that the protein was made up of a single polypeptide having approximately a molecular weight is 26kDa (Fig.4).

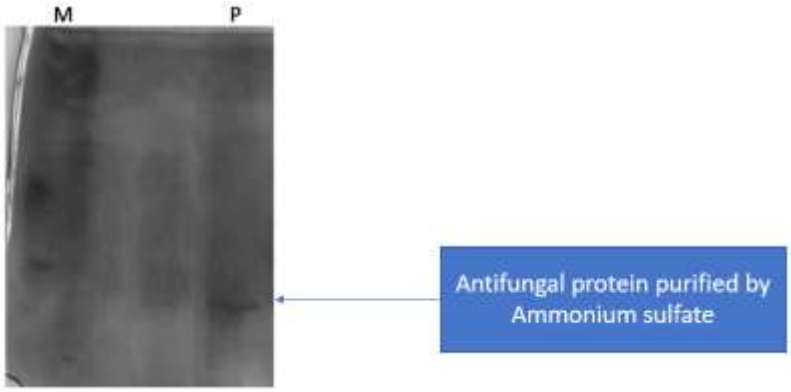

FIGURE 4. SDS-PAGE analysis of antifungal protein produced by isolate here M indicates the Marker of proteins and P means Partially purified protein by ammonium sulfate method.

DISCUSSION

As the new fungal pathogens have emerged causing diseases in animals and plants. So, there is a need for new antifungal agents to control such pathogens, with this aspect our finding is that the isolated organism is a potent antifungal protein producer. Screening for antifungal protein-producing microorganisms with excellent activity and low toxicity is an emerging activity in the present day. AMPs could be an alternative to the conventional antibiotics to which microorganisms especially opportunistic pathogens developed resistance and are thus essential to control (20) Future research should focus on determining the antifungal spectrum and investigating antifungal processes. We anticipate that the strain or its antimicrobial compounds will aid in the biocontrol of agricultural diseases and will be used in agriculture and food safety. This study reveals that the antifungal protein is easily extracted in single-step purification by ammonium sulfate precipitation. This organism could be applicable as a biocontrol agent against plant diseases and food preservation methods. The quest for biologically safer and environmentally friendly alternatives rather than chemical pesticides is driven by a growing awareness of environmental preservation and health concerns. Biocontrol solutions are more popular now than ever before as environmental worries about the effects of pesticides have increased. An alternative approach might include employing specific antagonistic microorganisms to biologically control of various things. The publication on the novel protein can provide additional information on Bacillus species. The protein purified from isolated bacteria may be a promising biocontrol option against pathogenic fungi.

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