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Article

Analyzing the Impact of a Formula Including a Partial Purified Aspergillus Niiger Protease

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Abstract: Aspergillus niger protease has attracted the attention of experts of environmental biotechnology since fungi can grow on low-cost substrates and secrete huge quantities of the enzyme into the culture medium. In this study, the samples of *Aspergillus niger* were collected from bread and onion, and then identified and screened for protease production using of skim milk agar. The production of media have followed components in gm/100 ml –casein 1g; yeast extract 0.6 g; urea 0.6g ; $NH_4H_2PO_4$ 0.6g; $(NH_4)_2SO_4$ 0.6g; NH_4Cl 0.6g; DDW 100 ml; glucose 1gm; and pH 8.5 for protease production , the produced protease in crud extract was partial purified with solid $(NH_4)_2SO_4$ was added to it by continuous mixing on ice bag (80% saturation) and the protease activity was 640 AU/ml with specific activity of 1280 AU/mg. The formula contained the partial purified protease was prepared, the protease produced from *A. niger* applied in formula was investigated by using skim milk agar ,wells contains the protease-containing formula has shown a significant clear zone and this ensure the activity of this formula.

Keywords: Protease ; Skim milk agar; Iraq.

1. Introduction

Proteolytic enzymes like proteases are included in a sub-class of the enzymes hydrolases. These enzymes cause a breakdown for proteins into smaller peptides and amino acids by catalyzing the peptide bonds. Proteases of the subtilisin group are used for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses to speed up healing process by producing anti-inflammatory response in patients have been reported. It is possible to use protease as a therapeutic agent for the treatment of pulmonary emboli and degradation of elastin and collagen [1]. The purified protease from bacteria and fungi could be used for various purposes like antibacterial activity against clinical pathogens as well as it degrade slime and biofilms to limit gram negative bacteria [2]. *Aspergillus niger* is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans [3]. The fungus is generally considered as a good strain for the production of protease enzymes. They are simple to be regenerated from mold fermentation broth of the genus *Aspergillus*, *Penicillium*, and *Rhizopus*; and are very valuable for protease production [4]. Fungal like *A. niger* protease has attracted the attention of experts of environmental biotechnology since fungi can grow on low-cost substrates and secrete large quantities of the enzyme into the culture medium. Production of fungal protease has advantages over bacterial protease as mycelium may be easily detached by filtration [5].

2. Materials and Methods

2.1. Sample collection

Isolates of *A. niger* were obtained from bread, onions and soil. The soil samples were seeded in sterile distilled water, serially diluted and inoculated on Potato Dextrose Agar (PDA) or samples inoculated on Sabouraud dextrose agar (SDA) with chloramphenicol incubated in 25°C for 12 day for fungal isolation. The bread and onions were moistened and kept at room temperature to develop fungal growth, which were plateand on PDA. Fungal growth suspected to be *A. niger* based on macroscopic observation (carbon black or dark brown conidia) were further sub-cultured on fresh PDA plates. The isolates that exhibited the characteristics of *A. niger* growth; initially white and quickly turning black, were subjected to microscopic observation with reference to the manuals of previous report [6]. The colonies were identified according to their color, shape, consistency and color of reverse plateand [7].

To confirm the diagnosis, wet mount smears prepare by take loopful from the fungal growth and mixed with 1-2 drop from lacto phenol cotton blue stain on the slide that examined under light microscope at X10 and X40 objective lens, to detect the structures of the fungus, which include the hyphal and conidial elements that appeared to recognize the strains and after recognizing the fungus re-inoculate in new plate for purification [8].

2.2. Screening of protease producing fungi

Skim milk agar medium (Oxoid, England) is used for primary screening of protease producing fungi by mixing 25 g of nonfat dry milk was mixed with 250 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 121ºC for 15 min. The pH of the medium adjusted using the pH meter (OMEGA, England), and maintained at a pH of 6. The suspension of 2.5% agar (Oxoid, England) used for solidification were autoclaved at 121ºC for 15 min. For plating, skim milk and agar solutions were held in a water bath at 50ºC and then the skim milk was poured into the agar flask and mixed thoroughly. To restrict the bacterial growth 1 mg/1000 ml ampicillin (Duchefa Brochemie B.A, Netherlands) was added to the media. The skim milk agar was poured quickly into plates and kept at 4ºC until be used [9]. The pure culture isolates were streak on the skim milk agar plates and incubated at room temperature for four days. Subsequently, the appearance of clear zone in the medium around the colony indicates protease activity and was selected for this study [10].

2.3. Production of protease enzyme

The selected isolates of the fungi used in the Fermentation were carried out in Erlenmeyer flasks (250 ml) containing the production media for protease, which having the following components in g / 100 ml: casein 1g, yeast extract 0.6 g, urea 0.6g, $NH_4H^2PO_4$ 0.6g, $(NH_4)_2SO_4$ 0.6g, NH_4Cl 0.6g, DDW 100 ml, and glucose 1g, at a pH of 8.5. In Production media, 2 ml of spore suspension of selected isolates was inoculated, and the flasks were kept on shaker at 120 rpm for 72 hrs incubation [11].

2.4. Plate assay by agar well diffusion method

Proteolytic activity of crude enzyme from selected *A. niger* was checked by agar well diffusion method on skimmed milk agar plate. Equal amount of crude enzyme (0.5µl) was dispensed in wells bored in skimmed milk agar plates that then incubated at room temperature for 48 hrs. Zone of proteolysis was observed as clear zone of hydrolysis around agar well [12].

2.5. Partial purification of protease

After centrifugation of filtrate at 10,000 rpm for15 min, supernatant was collected, and the solid $(NH_4)_2SO_4$ was added to it by continuous mixing on ice bag (80% saturation). Then, the mixture was kept for overnight at 4ºC for precipitation of protein, and then, centrifuged at 10,000 rpm for 20 min. Pellet was collected and dissolved in 15 ml phosphate buffer, and subjected for dialysis using the dialysis bag and magnetic stirrer. A partially purified enzyme proteolytic activity was determine by agar well diffusion method [11].

2.6. Preparation pharmaceutical formula including partially purified protease

The formula contained the partial purified protease was prepared asfollow modified method [13]:
a) Totally, 0.1 gm of methyl paraben was dissolved in 1ml of ethanol (70%).
b) A 49 ml of olive oil was gradually added with continues mixing.
c) A 5 ml of partially purified protease with an activity of AU/ml was added with mixing until homogenization.
d) A 50 gm of white petroleum vaseline was added gradually with continuous mixing to homogenization.
e) The prepared formula was stored in close container at 4ºC

3. Results

3.1. Culture

The isolates of *A. niger* were appeared as a woolly, white to yellow in color and the vesicle was appeared as globular brown to black due to conidia head turn to dark brown- black while in reverse white to tan (Figures 1, 2).

3.2. Screening for protease production

The fungal isolates were screened for their ability to produce protease enzymes. Majority of the isolates showed protease production activity. The maximum production observed after 96 hours. Based on the skim milk agar experiment for protease detection the highest clear zone on skim milk agar was from bread sample protease activity increased after 96 hrs of incubation. All the protease producing isolates showed clear zones around their colonies with variation in the diameters and the highest clear zone was from bread sample (Figure 3).

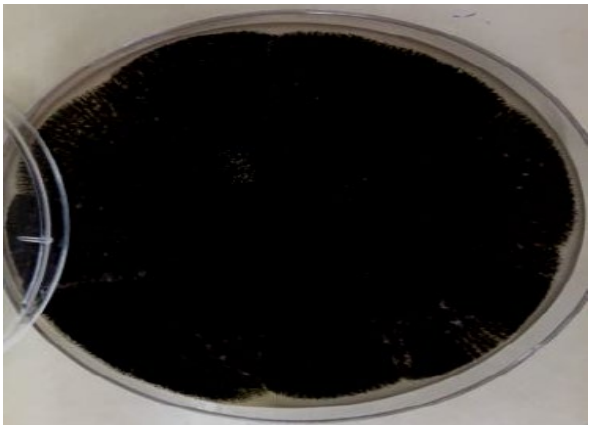

Figure 1. Morphological appearance of selected *A. niger* on PDA plate

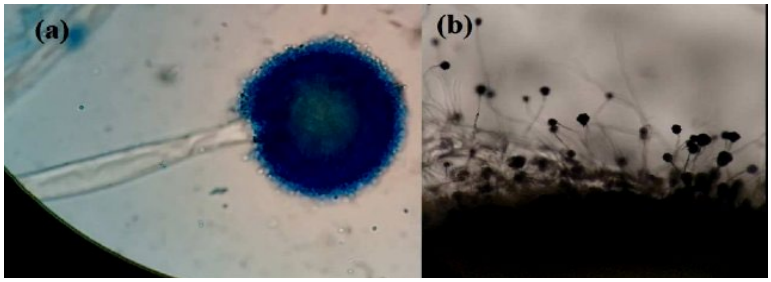

Figure 2. Microscopic view (40X) of (a): *Aspergillus niger* stained with Lactophenol cotton blue; (b): Slide culture

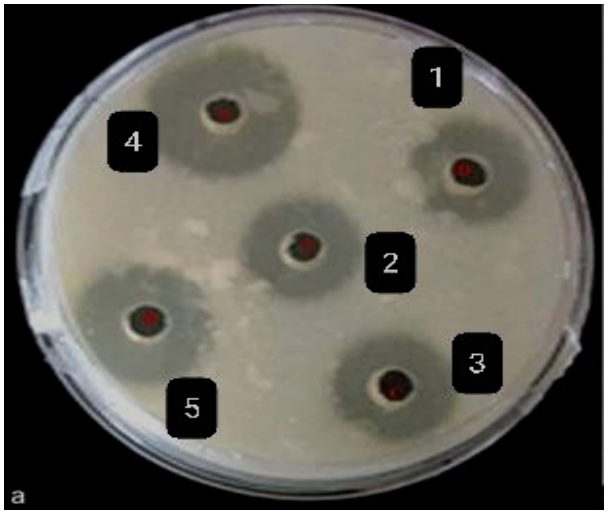

Figure 3. Well diffusion method 1,2 ,3 crud enzyme(triplicate). 4,5partial purified protease (duplicate)

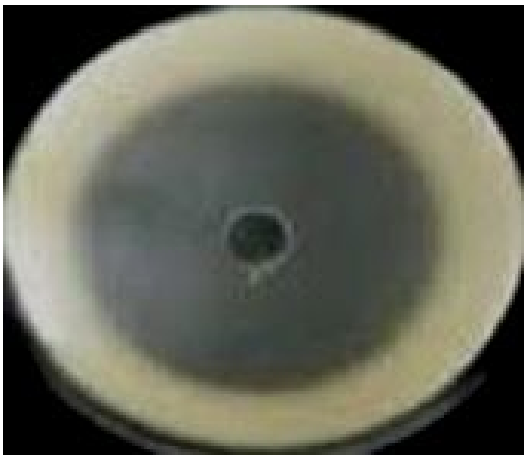

Figure 4. Clear zone on skim milk agar result from formula containing partial purified protease

3.3. Partial Purification of Protease

The studied protease produced by *A.niger* was precipitated from the culture supernatant by saturation with 80% of ammonium sulfate followed by dialysis to remove salts and impurities. Based on the results maximum protease precipitation, the protease activity was 640 AU/ml with specific activity of 1280 AU/mg. the partial purified protease saturated with 80% ammonium sulfate was assayed using skim milk agar (Figure 3).

3.4. Evaluation the activity of the formula including protease

The activity of protease produced from *A. niger* applied in formula preparation was investigated by using skim milk agar [13]. Wells contains the protease-containing formula has shown a significant clear zone. Some of points have surely illustrated the efficiency of the formula, liberating to external environment from the formula, without interface with protease or any of formula's component and increased of clear zone in skim milk agar when the formula used in well diffusion method as in (Figure 4) above.

4.Discusion

Six fungi isolates were isolated from soil samples. The findings of culture were in agreement with that observed by other study [14]. For all the 6 isolates, qualitative (zone of inhibition) protease assays was done. The result of protease production after using production media and used clear supernatant that show increased in clear zone in skim milk plate when used well diffusion method [12].. The isolate was checked for quantitative test of extracellular protease in liquid medium, then the extracellular protease was pricipated by ammonium sulfate by using many ratio of ammonium sulfate ratio and found the 80% was the best ratio for protease precipitation from this fungus isolate and give 640 AU/ml with specific activity of 1280 AU/mg. Bellaouchi et al. (2021) concluded that *A. niger* showed the ability to produce several extracellular enzymes and can be used in the valorization of different agroindustrial residues [15]. The precipitative protease was used within formula that checked their activity within this formula and found increased of enzyme activity by increased of inhibition zone and as a result used this protease - formula in medical application. These findings were in agreement with that observed by other studies [16-18-19-20].

5. Conclusions

The high rates of protease activity from fungal samples espacialy *Aspergillus . niger* and use this partial purified prtease in formula then assayed the protease activity within formula give high inhibition zone in skim milk as screening test. The ability to produce extracellular protease by means of formation of clearing zones around the fungal growth in skim milk ager plates. All the protease producing isolates showed clear zones around their colonies. Furthermore, the best isolate produced a maximum amount of extracellular protease was identified, the potent fungi was identified based on morphological characterizations

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