Original Article

Evaluate the effect of the formula containing partial purified of protease produced from Bacillus spp

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Abstract

In this study was used Bacillus spp. isolate after collection and identification and screening as the best isolate for protease production by using skim milk agar as screening media then the isolate that give the highest proteolytic activity was chosen and the protease was partially purified by the ammonium sulfate and sephadex G-50 to give activity 320 and 640 U/ml subsequently , then the activity was examined in vitro with formula by using skim milk agar as a screening media to detect of protease activity within the formula the result proved increasing of protease activity by increasing of clearing zone around well of well diffusion assay of skim milk and can used this formula for medical application.

Keywords: Bacillus, Protease, Skim milk, Antimicrobial

INTRODUCTION

The production of proteases by microbes is crucial to several industries, including waste management, the detergent and tanning sectors (1). A class of enzymes known as proteases has the catalytic activity of hydrolyzing protein peptide bonds and dissolving them into polypeptides or free amino acids (2). Genus-specific rod-shaped, gram-positive, aerobic and endospore-forming bacterium that having greatest diversity in soil are Bacillus. One of the several extracellular enzymes produced by Bacillus is proteases. Proteases are produced by a number of Bacillus species, including B. subtilis, B. sterothermophilus, B. mojavensis, and B. megaterium (3). In medical field, proteases have therapeutic potential in the composition of ointments, non-woven tissues, and gauze (4). Proteases have unique therapeutic capabilities that are useful in creating numerous medications against fatal diseases, including those that fight cancer, bacteria, and inflammatory infections, as well as those that dissolve blood clots, among many other uses (5).

MATERIALS AND METHODS

Sample collection

Bcillus spp. are widely distributed in soil and the aquatic environment so the samples of bacteria collected and obtained from 50 samples 10 isolates from different area humid and dry soil and 2 isolate from water samples. The samples were cultured on nutrient media incubated in 37ºC for 24 hrs; the samples were identified by conventional biochemical methods (6).

Sample identification

The samples were homogenized in PBS 0.05 M, pH 7.2, serially diluted in the same medium, spread out on nutrients, and incubated at 30ºC for 24-48 hours. On the isolates, several of biochemical experiments were carried out, that prove if the isolates belong to the genus Bacillus bacteria as the method of (7).

Screening of protease-producing Bacillus spp.

Every Bacillus species, A 2% skim milk 1% bacto agar dish, was used for the inoculation of the isolate, which was then incubated at 37°C for 24 hours (8).

The increasing production of Protease

After being chilled, glucose was sterilized individually and aseptically introduced to the flasks holding the liquid medium. The following ingredients were present in the culture media used to make the protease: glucose (0.1%), peptone (1.0), yeast extract (0.02), MgSO4 0.01%, CaCl 2.01%, and K2HPO4 (0.05%) (pH 7.0). The precultures were grown for 18 hours in nutrient broth medium (0.8% w/v). After that, overnight cultures with an OD600 of 0.3 were injected at 1% in media for producing enzymes(150 mL in 500 mL Erlenmeyer flasks), and they were then incubated for 24 hours at 37 °C in a shaking incubator(150 rpm). Supernatants from the centrifuged cultures (6000 rpm, 10 min) were utilized to measure the proteolytic activity (9).

Protease's partial purification

By ammonium sulphate (75%) precipitation and dialysis, the crude protease extract of a chosen isolate of protease-producing Bacillus spp. was partially purified. Centrifugation at 12,500 rpm for 20 minutes at 4 °C was used to collect the precipitate, which was then dissolved in 0.01 M PBS buffer (pH 7.0). The amount of protein was calculated as in the method of (10), by using BSA as the standard protein after the solution had been dialyzed against the same buffer at 4 °C for 8 hours, crud protease was produced and put onto a 3 *20 cm column of sephadex G-50 gel filtration. PBS (0.1 M, pH 7.2) elution was carried out using fractions of 3 ml and a flow rate of 36 ml/hour. At 280 nm, the absorption was observed. Protease activity was evaluated in the fractions. Protein concentration and protease activity were measured after mixing the fractions that indicated protease activity in one tube (11).

Preparation of pharmaceutical formula including protease

A- An aliquot of 4 gm of carbopol 941 was dissolved in 95 ml D.W and then mixed very well using magnetic stirrer. Next, 0.1 g of methyl paraben was added. The mixture was left soaking for 24 hrs. Then, the partial purified protease was prepared in the optimized medium and mixed well. Next, the final volume of the formula was completed to 100 g.

B-All compounds included the partial purified protease AU/ml, production medium and other formula chemicals were mixed together for 1hr.

C-Sufficient amount of triethanolamine was added subsequently to obtain gelling formula texture and pH was adjusted to pH 6.4.

D-The formula was stored in a close container at 4℃ (12).

Agar Well Diffusion Method for Plate Assay

Agar well diffusion was used to test the proteolytic activity of a partially purified enzyme from a chosen Bacillus spp. on a skimmed milk agar plate. In wells drilled in agar plates with skimmed milk, an identical quantity of crude enzyme (0.5l) was applied. For 24 hours, plates were incubated at room temperature. Around the agar well, a definite zone of hydrolysis representing the zone of proteolysis was seen (13).The activity was assayed with enzyme alone and enzyme within formula to check the activity of protease within formula.

Results and discussion

Themorphology shape of Bacillus spp. were seen as coral/rooted-like edges, crooked edges, flat edges that look like cotton and flower-like edges resembling a colony of mold. And show as Gram-positive, aerobic, rod-shaped endospore-producing (3), as shown in figure (1).

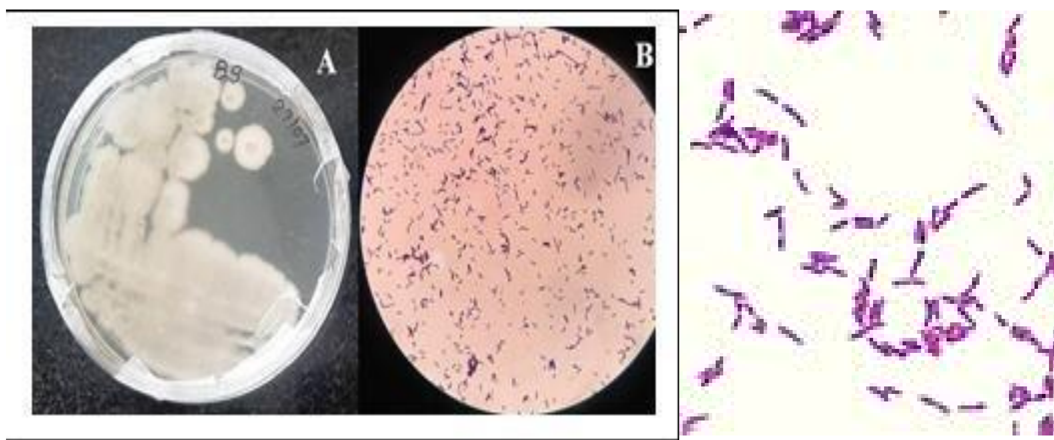


Figure 1-A-The morphological and B-microscopic view of Bacillus.spp

Table- ١ : The Biochemical tests.

Characteristic	phenotype
Growth temperature	٥-٥٠ºC
Growth ph	٥-١١
Nacl tolerance	١٠٪
Oxidase	+
Catalase	+
Indole production	-
Voges-Proskauer	+
Citrate utilization	-
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
Galactose	+
Fructose	+
Mannose	+
Amylase	+
Urea	-
DNase	-

The results of biochemical assays as shown in Table -1 that prove the bacteria belong to the genus of Bacillus.spp, The most prevalent organisms in soil are gram-positive, aerobic, rod-shaped endospore-forming bacteria of the genus Bacillus and adapt easily to diverse habitats (14). After isolation of bacteria then cultured on skim milk agar and closed the best isolate that produced highest clear zone on skim milk and used in present study as shown in figure 2.

Figure - 2 Bacillus. spp. culturing on skim milk

The result that show the ability of the isolate by creating clearing zones around the bacterial growth on skim milk agar plates, extracellular protease is produced. Then the isolate was cultured on production media that used and then leading to increase the protease activity to reach /U/ml and chose this media as the best productive media. By saturating the culture supernatant with several levels of ammonium sulfate, ranging from 20 to 90%, and then dialysis to remove salts and contaminants, Bacillus spp. protease was precipitated from the supernatant. Maximum protease precipitation, according to the findings, was attained at a 75% saturation level. With a specific activity of 1066.66 AU/mg, the protease activity was 320 AU/ml. Following that, sephadex G-50 was loaded with the precipitated protease. Each absorbance at 280 nm was conducted, and the separation profile showed two distinct peaks. Figure (3) shows that fractions 31 to 36 had protease activity. In order to get 3 ml of active fractions, sucrose was used to concentrate them. The partial purification of the protease's activity and specific activity were then estimated in accordance with the purification's examples (Table 2).

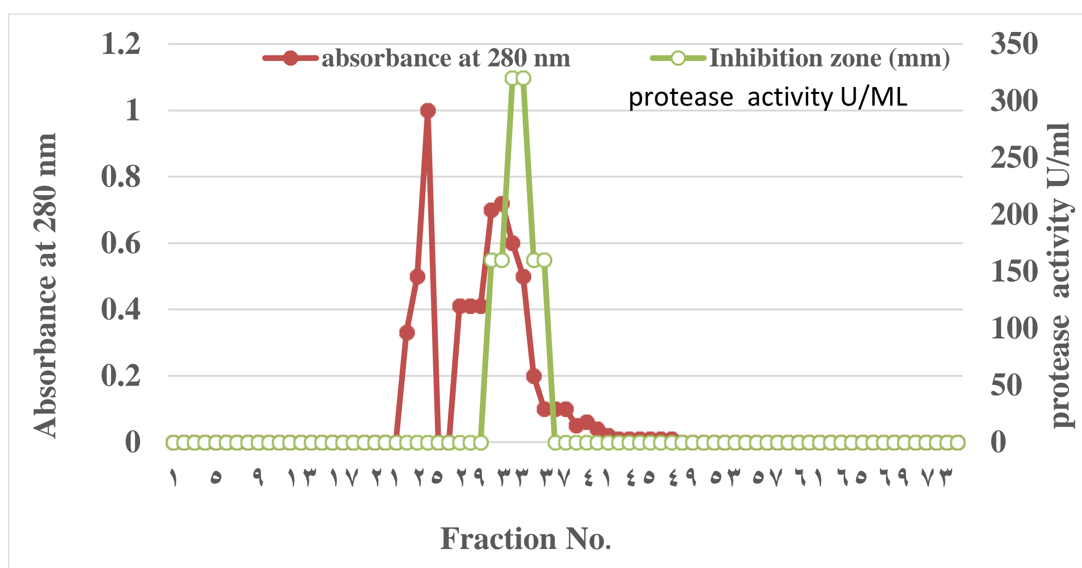
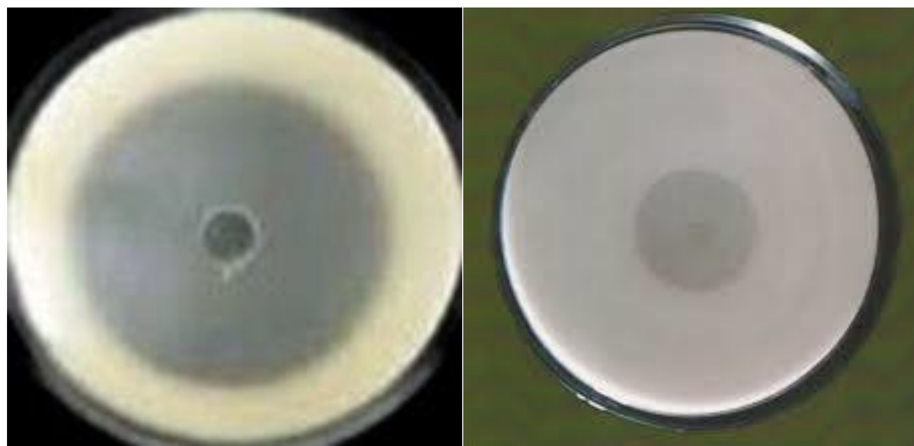


Figure ٣. Purification of protease produced by *Bacillus .spp* by Sephadex G-٥٠ column (٣ × ٢٠ cm). Column was equilibrated and eluted with sodium phosphate buffer, pH ٧ at a flow rate of ٠.٦ ml/min

Table ٢. Summary of purification of protease from crude culture filtrate of *Bacillus .spp*

Purification steps	Volume ml	activity) U/ml)	Protein concntration mg/ml	Total activity U	Specific activity mg/U	Yield %	Fold purification
Crud extract	100	160	0.5	16000	320	100	1
Ammonium salt precipitation 75%	10	320	0.3	3200	1066.66	20	3.33
Gel filteration sephadex G 50	3	640	0.2	1920	3200	12	10

The partial purified protease was introducedin formula and examined in vitro.The activity of formula containing protease was examined by using skim milk and give increasing of the zone around well in well assay on skim milk and this result ensure the efficiency of formula that containing partial purified protease without inhibition of protease activity as shown in figure (4) and as a result can used this formula in treatment of cutaneousinfection because The proteases have unique therapeutic capabilities that are useful in creating a wide range of medications against microbial and inflammatory infections as well as for a variety of other purposes (5).



B- A-

Figure (4) A-The partial purified protease in well plate assay on skim milk agar, B-The partial purified protease within formula in well plate assay on skim milk.

Conclusion

Bacillus.spp bacteria are important group of bacteria that usfual in many application like medical , industral and pharmacetical field and widely used because of ability to produce many of antimicrobial agent that applying as formula or as a agent for the treatment.

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