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Primary and Secondary Screening of *Pseudomonas aeruginosa* for Protease Production

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Abstract: The current study was aimed for inhibition of purified protease produced by *Pseudomonas aeruginosa* using alcoholic extract of *Conocarpus lancifolius* leaves. A total of one hundred forty-six of isolates of *P. aeruginosa* that were isolated and identified by microscopic and biochemical tests were fifty-one isolates submitted to primary and secondary screening techniques in order to choose the qualified *P. aeruginosa* isolate for protease synthesis. Among these isolates, forty-seven isolates with the show hydrolysis zone on skim milk media (primary screening) were chosen six isolates for secondary screening. The result revealed that *P. aeruginosa* P51 had the highest ability to produce the enzyme (specific activity 15.9 U/mg protein). The optimum conditions of protease production by the selected isolate in submerged fermentation by using tryptic soya broth medium as best substrate, temperature 37°C and pH 8, after 48 h of incubation. In addition, the study was included extraction of *C. lancifolius* crude by using 80% ethanol. It was concluded revealed a significantly decreasing in specific activity of protease (from 15.9 to 4.2 U/mg) after treated it with 0.4 µg/ml of alcoholic extract of *C. lancifolius* crude extracts.

Keywords: *Conocarpus lancifolius*, Protease, Plant extract, inhibitory activity.

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Introduction

Microorganisms are responsible for the production of a wide variety of products, the nature of which is determined by the metabolic processes they engage in and the substrates they feed on. Many of these products are thought to be naturally occurring poisons. They may either be released into the growing medium, in which case they are referred to as exotoxins, or they may collect within the cells of the host, in which case they are known as endotoxins. Slime, exotoxin, interotoxin, hydrocyanic acid, pigments, and phytotoxic factor are some of the extracellular toxins that *Pseudomonas aeruginosa* is capable of releasing (1)

Pseudomonas aeruginosa is an opportunistic pathogen. It can cause death and morbidity in immunocompromised patients or those with cutaneous, respiratory, urinary, or nosocomial infections. Pathogenic microorganisms can resist disinfectants and medications. (1,2).

It was extracellular protease is important to its pathogenicity. (3), wherein the peptide bonds found in proteins are hydrolyzed by this enzyme, resulting in the formation of polypeptides or free amino acids. Proteases are capable of causing a wide

variety of deleterious effects, including necrosis of tissue and hemorrhage (4).

Materials and methods

Collection, isolation and identification of samples protease-producing isolates

Patients of different ages, both male and female, from different teaching hospitals in Baghdad city were asked to give blood samples. A total of 146 samples were taken (October 2021 to November 2021). These samples came from wounds, burns, ear infections, and even infections in the urinary tract. To figure out what these samples were, they were grown on MacConkey agar, blood agar, Pseudomonas agar, and Cetrimide agar. A few biochemical tests and a microscopic look (11). The identified *P. aeruginosa* isolates were prepared for screening experiments.

Primary (qualitative screening) of *P. aeruginosa* isolates for protease production

Most of the 51 *P. aeruginosa* isolates were tested on a medium of skim milk to find the ones that made the most protease. A culture of bacteria was grown in skim milk agar for 24 hours at 40°C. The colony's inhibition zone was a sign that protease was being released(12) .

Secondary screening (Quantitative screening) of *P. aeruginosa* for protease production

In order to get a better understanding of the potential of six isolates, quantitative testing was performed. Two hundred and forty-five milliliters of a bacterial isolate that had been activated overnight were placed in a 25 milliliter volume of casein-pepton broth and incubated at 37 degrees Celsius for 24 hours. The crude enzyme was centrifuged at 3500 rpm for 20 minutes following incubation. Then, the supernatant was analyzed for its specific activity, protein content, and enzyme activity (13).

Protease activity assay

A 1.8 ml of 1% casein solution was prepared, after that, tubes were placed in water bath at 50^0 C for 5 min. A 0.2 ml of enzyme was incubated with casein solution at 37 ^{0} C for 30 min. A 2 ml of 5% TCA was added to stop the reaction, then the mixture was centrifuged at 6000 rpm for 15 min. The control was prepared by mixing (0.2 ml enzymatic solution, 1.8 ml of 1 % casein solution and 3 ml of 5 % TCA). The absorbance of the supernatant was measured at 280 nm. The activity of enzyme was calculated based on the degradation of casein into peptides and amino acids by using the formula (14):

$$Enzymatic\ activity\ \left(\frac{U}{ml}\right) = \frac{\text{Absorbency at 280 nm}}{0.2 \text{ x } 30 \text{ x } 0.01}$$

Where 30 referred to time of the reaction (minute), 0.2 referred to the volume of the adding enzyme solution in ml and 0.01 referred to the amount of enzyme to increase the absorbance in amount 0.01 per minute under assay condition.

Protein concentration assay

Use two tubes per concentration. A 0.4ml of tris-HCl pH 8 and adding sample 0.1 ml was added to each tube. A 2.5 ml of coomassi blue stain G-250 was added to each tube then mixing, the mixture was left to 5 min. For preparation of blank, 0.5 ml of tris-Hcl and 2.5 ml of coomaai blue stain G-250 were added. Reading absorbency per

concentration at wave length 595 nm by spectrophotometer and specific activity was measured as described by using the following formula (15):

$$Specific\ activty\ \left(\frac{U}{mg}\right) = \frac{Enzyme\ activity\ (U/ml)}{\text{Protein concentration}\ (mg/ml)}$$

Results and discussion

Isolation and Identification of *Pseudomonas aeruginosa*:

The collected 146 samples were cultivated on Blood and MacConkey agar to distinguish bacteria if they were gram positive or negative. The results revealed that some of these samples were grown on MacConkey agar only, while the others were grown on both media, which means that they are gram negative bacteria. All isolates were grown on MacCkonky agar which considered as a differential medium for distinguish the lactose fermented and non-fermented bacterial isolates(16), so that the gram negative have grown as a pale shape because of these bacteria were non-lactose fermented bacteria. Then, these isolates were re-cultured in Pseudomonas agar as a selective medium for *Pseudomonas* genus, and incubated at 42°C for being an important diagnostic characteristic for *P. aeruginosa* than other species of this genus. The results showed that 51 isolates have ability to grow in this medium at this temperature, and the colony demonstrated as a regular ring shape and creamy color.

These isolates were re-cultured in Cetrimide agar to confirm the diagnosis,

where *P. aeruginosa* differ from other *Pseudomonas* species by growth on the selective medium (Cetrimide agar) (17), all isolates were grow on this media as greenish-yellow color colonies, which confirmed the *P. aeruginosa* sp. due to they have the ability for resist the cetrimide material which is considered as toxic material for other bacteria resulting in releasing nitrogen and phosphorus from bacterial cells more than *Pseudomonas* sp.

Protease production

Protease production experiments (Table 1) showed that 47/51 (92.2%) of *P. aeruginosa* isolates were able to produce protease in skimmed milk agar (1%), where these results indicated by seen zone lysis around the colony in varying degrees ranged from 6 to 35 mm. this variance in zone lysis may due to the diversity of isolate sources and culture conditions, while 4/51(7.8%) of isolates didn't show any zone lysis around their colony. These results agree with Onal *et al*., (18) who found that most clinical isolates represent 93% which were able to produce protease (18). In addition, Younis *et al*., (19) who found that only (34%) of *P. aeruginosa* isolates were positive to protease (19). Protease is considered one of the important virulence factors and it plays role in lysis tissue protein such as elastin and collagen, and helps bacteria to invade infected tissues, especially in people with burns, also it acts to protect bacteria from body defenses (20).

Table (1): Production of Protease by *P. aeruginosa* isolates in skimmed milk agar

No. of *Isolates*	Protease Production	No. of *Isolates*	Protease Production	No. of *Isolates*	Protease Production
P1	++	P18	+	P35	-
P2	+	P19	+	P36	+
P3	+	P20	+	P37	+
P4	++	P21	+	P38	+
P5	+	P22	++	P39	+
P6	+	P23	+	P40	+
P7	+	P24	+	P41	+
P8	+	P25	+	P42	+
P9	+	P26	+	P43	+
P10	+	P27	+	P44	+
P11	-	P28	+	P45	+
P12	+	P29	+	P46	-
P13	+	P30	+	P47	-
P14	+	P31	++	P48	+
P15	+	P32	+	P49	+
P16	+	P33	+	P50	+
P17	+	P34	++	P51	++

P1- 51 : *P.aeruginosa* isolates.
++: High Product of Protease (More than 18mm. of Zone lysis).
+: Middle Product of Protease (Less than 18 mm.)
- : No product.

Secondary screening

Six isolates have been chosen for secondary screening to select the highest protease-producing isolate. The results showed that the specific activity of protease produced by these isolates ranged between 7.5-15.9U/mg (Table 2). Gençkal and Tari (21) referred that the differences in the enzyme production for each isolate might be because of the variation in codes of gene result in variation of enzyme synthesis (21,22). Evaluation of protease activity depends on the enzyme ability to casein degradation, for that, casein was used in medium for the detection the protease produced by microorganisms. The method for measuring the activity of protease based on the precursivity of casein is one of methods widely used for determining the efficacy of the enzyme in the bacterial culture (5). Depending on the results obtained from the study, *P. aeruginosa* P51 isolate showed the best efficiency for the production of protease enzyme.

Table (2): Specific Activities of protease produced by *pseudomonas aeruginosa*

Number of P. aeruginosa	Enzymatic specific activity(U/mg)
p1	7.5
P4	13.1
P22	9.6
P31	8.2
P34	12.8
P51	15.9

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