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Production of Protease Mutant *Pseudomonas aeruginosa* isolated form clinical samples

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Abstract— During this study, a total 150 clinical samples were collected from different clinical cases (burns, wounds, otitis media and UTIs) from (3) hospital in Baghdad and Bald general hospital during a period from 1/7/2017 to 1/12/2017 for isolation of *Pseudomonas aeruginosa.* Results revealed after microscopic examination and culture on different selective and differential media, then identified by Api20E that give only 92 isolates was related to *Pseudomonas aeruginosa*, later only ten of these *p. aeruginosa* isolates was identified by vitek2 system. For detection the ability of *Pseudomonas aeruginosa* isolate for protease production, 36 isolate were secerned only for protease production on skim milk agar. Results revealed that all 36 isolates were protease producer and among them *p. aeruginosa* p3 isolate was most efficient one that give hydrolysis zone reach to (28mm) and have specific activity (o.123 U/mg protein) in culture filtrate. Upon this *p. aeruginosa* p3 isolate was selected to enhance its ability in protease production by random mutagenesis. It was subjected to physical mutagenesis by UV irradiation and chemical mutagenesis by using NTG. Results showed that an over protease producing mutant symbolled *P. aeruginosa* p3 isolates after mutagenesis with UV irradiation for different time (0,10, 20, 30, 40, 50, 60, 70, 80, 90) sec. that the LD_{90} reach after 70 sec. and the enzyme specific activity in its crude filtrate was 0.431 U/mg protein in comparison to the wild type (0.132U/mg protein). On the other hand, mutant *P. aeruginosa* p3 isolate was obtained after chemical mutagenesis with NTG for different time (0, 20, 40, 60, 80, 100, 120) min. respectively it characterized with its faint ability in protease production. The enzyme specific activity in its crude filtrate was 0.0894 U/mg protein in comparison with the wild type 0.123 U/mg protein.

Index Terms— Minimum 7 keywords are mandatory, Keywords should closely reflect the topic and should optimally characterize the paper. Use about four key words or phrases in alphabetical order, separated by commas.

1 INTRODUCTION

THe bacteria *Pseudomonas aeruginosa* is a Gram-negative non-spore forming, straight or slightly curved rods. The strains of bacteria measuring (0.5 to 0.8) µm in length and (1.5 to 3.0) µm in width, they are typically motile by means of one or more polar flagella (Arora *et al.,* 2005).

Pseudomonas aeruginosa bacteria is wide spread in nature inhabiting soil, water, plant, animal and human. The bacteria have minimal nutritional requirement and able to tolerate a wide range of environmental conditions (Fogle, 2012).

Pseudomonas aeruginosa is an opportunistic pathogen that is rarely caused disease in healthy persons, but its often colonize immunocompromised patient, like those infected with cystic fibroses and AIDS (Karimi *et al.,* 2002).

The bacteria have numbers of virulence factors that are either cell associated or extracellular factors and have an important role in the pathogenesis of bacteria in human and experimental studies in animal (Girard and blomberg, 2008).

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (Deng *et al.*,2010). They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al.*,2007 and Jellouli *etal.*,2009).

Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Neutral proteases are mainly of plant origin. While proteases having optimum activity at pH range of 8 and above are classified as alkaline proteases produced from microorganisms.

Proteases produced from microorganisms play important role in several industries example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. (Gupta *et al.*,2002). Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009).

The protease enzymes have an important role in the pathgenesis of *Pseudomonas aeruginosa* that lead to degradation in the structural protein and facilitated bacterial adhesion and infection (Lucas *et al.,* 2006). Also these enzymes responsible for ocular eye infection and acute lung injury (Jayati and Rinta , 2006).

The extracellular proteases of *Pseudomonas aeruginosa* (alkaline protease, protease IV and elastase) may increase in proportion to the duration of tissue colonization and are considered to be important virulence factors in several human infections caused by this opportunist pathogen (Shaw *et al.,* 2004). Also Proteases play a crucial role in numerous pathologic processes such as Arthritis, tumor invasion and metastasis, also infection and development of degenerative diseases have been linked with the involvement of one or more proteolytic enzymes (Sally *et al*., 1999).

This study was aimed to detect effectivity and pathogenisity of extracellular protease that produced from *pseudomonas aeruginosa* in mice model by made histopathological section of liver and kidney.

1.1 Virulance factors of psedomona aeruginosa

This bacterium has many virulence factors such as Protein F, Lipopolysaccharide, appendeges (pilli & flagella), biofilim, exotoxin A, protease, Elastease A, Elastease B, Protease IV, pigments, Lekocidin.

1.2 Physical mutagens

Any agent that damages DNA can lead either to the death of the organism or survivors to mutation. Many types of physical mutagen have been used to generate mutation. The higher energy rays such as X-rays require expensive apparatus and safety equipment and are not really suitable for routine use in a microbiology laboratory. In addition, they produce an excessive amount of chromosomal damage that is not easily repaired by the Mo. Ultraviolet irradiation on the other hand is easily controlled (although eye and skin protection is necessary) and requires only comparatively in expensive equipment (Mitra, 1996). The principal effect of UV irradiation is the production of pyrimidine dimmers (commonly referred to as thymine dimmers). Where two pyrimidine residues are adjacent on the same DNA strand, the result of UV irradiation is the creation of covalent links between them.

1.2 Chemical mutagens

The natural rate of spontaneous mutation is much too low for convenient isolation of most types of mutants (apart from a handful of easily selected mutations such as antibiotic resistance). Ways must be found of enhancing that frequency. It is often possible to use *in vitro* mutagenesis or transposon mutagenesis (Paul and Leemor, 2007). but there are still many situations where chemical or physical procedures are preferred or essential. Many different chemical agents interact with DNA or with the replication machinery so as to produce alterations in the DNA sequence. Of these to understand are those agents that act by chemically modifying a base on the DNA so that it resembles a different base (Gerhardt *et al*., 1981). Alkylating agents such as ethyl methane sulphonate (EMS) and NTG are extremely powerful mutagens and the latter in particular is extremely hazardous to use. They act by introducing alkyl groups onto the nucleotides at various positions, especially the O6 position of guanine, and tend to cause multiple closely linked mutations in the vicinity of the replication fork (Jeremy and Simon, 2004).

2 METHEDOLOGY

2.1 Sample collection

A total of 150 clinical samples were collected from patients suffering from burns, wounds, otitis media and UTI infection in four hospitals AL-Imamein ALKadhimein Medical City hospital, Al-Yarmouk teaching hospital, alkarej hospital in Baghdad, and Bald general hospital during the period from 1/7/2017 to 1/12/2017. Samples from burns, wounds and otitis media were taken by a sterile swaps from a site of infection and placed in a ready a sterile transport media to maintain the swabs wet during transferred to laboratory, while a sterile container was used to take urine sample from patient suspected to have UTIs. Then samples were directly streaked on nutrient and MacConkey agar and incubated at 37°C for 24 hours.

2.2 Isolation of pseudomonas aeruginosa (Forbes et al., 2007).

After incubation, non-lactose fermenting colonies from Macconkey agar were subcultured on cetrimide agar, after 24 hours of incubation at 37°C, a loopful was streaked further on selective medium (king A, king B) agar plates and incubated at 37°C for 24 hours. Also selected isolated colonies from cetrimide agar were cultured on blood agar and incubated at 37°C for 24 hours, then colonies were subjected to further identifications.

2.3 Identification of *Pseudomonas aeruginosa*

2.3.1 Morphological Characteristic (Atlas *et al.*, 1995)

Colonies that able to grow on selective media were further identified by studying their morphological characteristics first by staining with Gram stain followed by examination of their (Gram reaction, shape, arrangement, spore formation) under light microscope.

2.3.2 Biochemical tests

suspected isolated colonies were subjected to a certain biochemical tests and as follow:

- **Catalase test**

A pure single colony from each bacterial isolates was taken from cetrimide agar and put on a clean slide, then a drop of hydrogen peroxide H_2O_2 was added and mixed together. The Presence of gaseous bubbles indicate a positive resul.

- **Oxidase test**

A few drops of a freshly prepared solution of tetramethyl phenylene diamine di hydrochloride was added on a filter paper, then a loopful of bacteria was picked up from cetrimide agar with a sterile wooden stick and smeared on a filter paper. The development of a violet or purple color within 10 seconds indicates a positive result.

- **Indole production test (Colle *et al.*,1996)**

Test tubes containing peptone water broth were inoculated with a fresh culture of each suspected isolates and incubated at 37°C for 24 hours. After that 0.5 ml of Kovac's reagent was added. The Presence of red ring on the surface of the medium indicates a positive result.

- **Methyl red (Atlas *et al.*, 1995)**

MR-VP broth in a test tube was inoculated with a fresh culture of each suspected isolates and incubated at 37°C for 24 hours, then 5 drop of methyl red reagent was added, mixed and reading the result. The appearance bright red color indicates a positive result.

- **Voges-Proskauer Test (Atlas *et al.*, 1995)**

Test tubes containing MR-VP broth were inoculated with a fresh culture of each suspected isolate and incubated at 37°C for 24 hours. Then 1ml of VP1 and 3ml of VP2 were added to 5ml of bacterial culture and mixed for 30 seconds. The formation of pink to red color indicates a positive result.

- **Urease Test (Maniatis et al., 1982)**

Test tubes containing urease medium were inoculated with a fresh culture of each suspected isolates and incubated at 37°C for 24 hours. Changing the color of medium from yellow to pink indicates a positive result.

- **Citrate utilization test (Atlas et al., 1995)**

Simmons's citrate slant was inoculated by streaking selected isolated colonies on the slant, then stabbing by loop in to bottom of it and incubated at 37ºC for 24 hours. Change the color of the medium from green to blue indicates a positive result.

- **Motility test (Atlas et al., 1995)**

Bacterial colonies were stabbed in a straight line on a semi-solid motility medium and incubated at 37°C for 24 hrs. Motile bacteria would grow and diffused around the line

- **Oxidative-fermentative test (Garrity, 2001)**

Two tubes of oxidative-fermentative medium were inoclated by stabbing half way to the bottom or ¼ inch from the bottom with a test organism. Overlay one of the tubes with 1 cm of mineral oil. This overlay prevents the diffusion of oxygen into the medium and creates an anaerobic condition in the tube. Incubation at 37°C for 48 hours is recommended for most gram negative rods.

- **Production of pyocyanine pigment (Kenneth, 2011)**

Selected isolated colonies from macConkey agar were streaked on cetrimide agar and King A medium for isolation of pseudomonas aeruginosa and detection of pyopcanine production, then incubated at 37ºC for 24 hours. The appearance of green colors on both mediums indicated a positive result.

- **Production of pyoveridine pigment (Kenneth, 2011)**

Selected isolated colonies from macConkey agar were streaked of King B medium and incubated at 37ºC for 24 hours. The appearance of fluorescence light on the plate under UV trans-illuminator indicate a positive result.

2.3.3 Identification of *Pseudomonas aeruginosa* isolates by API 20E system

Pseudomonas aeruginosa identification was carried out by sub culturing suspected isolated colonies from cetramide agar plates on API 20E micro tubes system. The system is consisting of 20 performance standard biochemical tests which contains an appropriate substrate that fixed to an impermeable plastic strip (gallery). The gallery contains 20 micro tubes that consist of a tube and a couple selection.

2.3.4 Identefecation of suspected bacteria by use VITek 2 system

VITEK 2 is a microbiological automated system that utilized a growth-based technology for identification of bacteria. This system is available in three formats (VITEK 2 compact, VITEK 2, and VITEK 2 XL) that differ in increasing levels of capacity and automation, but accommodate the same colorimetric reagent cards that are incubated and interpreted automatically. The VITEK 2system was first originated in the 1970s as an automated system for bacterial identification and AST and today has evolved into the VITEK 2 system, that automatically performs all of the steps required for identification of bacterial and AST after a primary inoculum has been prepared and standardized. The kinetic analysis in this system allows reading each test every 15 min. The optical system combines both the multichannel fluorimeter and photometer readings to record fluorescence, colorimetric signals and turbidity.

2.4 Screening for protease production from *pseudomonas aeruginosa* isolate

The ability of locally P. aeruginosa isolates in protease production was screened by two methods:

2.4.1 Semi quantitative screening (Sneath *et al*., 1986)

Single colonies from each *p. aeruginosa* isolate was streaked on cetramide agar medium and incubated at 37 °C for 24hrs, then a pure colony was taken and placed on a skim milk agar (This medium was prepared by dissolving 5 g of skim milk in 50 ml of distilled water, then sterilized by autoclaving at 121ºC for 5 min, also 2 g of agar was dissolved in 50 ml D.W and sterilized by autoclaving. After cooling to 45°C, they were mixed together before distributed in a sterilized plates and stored at 4°C until use) medium plate and incubated at 37°C for 24hrs. The ability of *p. aeruginosa* isolates in protease production was measured based on the presence of clear halo zone around colonies.

2.4.2 Quantitative screening of protease activity assay (Manachini *et al*., 1989):

Quantitative screening for protease production from local *pseudomonas aeruginosa* isolates was achieved by determining the enzyme activity and specific activity according to the following steps:

- -A volume of 100 µl of fresh culture of *pseudomonas aeruginosa* isolate was used to inoculate the production medium (as prepared by added 1g peptone, 0.5g beef extract, 0.5g NaCl that dissolved in 10 ml of D.W, the volume was completed to 100ml with distilled water, pH was adjusted to 7, then sterilized by autoclave at 121ºC for 15 min**,** poured in a sterile container and stored at 4Cº until use) in a conical flask and incubated in a shaker incubator (150 rpm) at 37ºC for 24hrs.
- -After incubation, the culture was centrifuged at 6000 rpm for 15 minutes, pellets were discarded and supernatants were taken and assayed for protease activity.
- -0.8 ml of the 1% casein solution was prepared as described in (2.1.4.3 B).
- -0.2 ml of enzyme solution was added to casein solution and incubated for 30 minutes at 37^{0}C.
- -The reaction was stopped by adding 1 ml of the 5 % TCA, then chilled in an ice bath for 10-15 min before the solution was centrifuged at 6000 rpm for 15 minutes.
- -The control test was prepared by adding 1 ml of 5 % TCA to 0.8 ml of 1% casein solution, then 0.2 ml enzyme solution was added to it.

The absorbance for the supernatant was measured at 280 nm using UV-VIS spectrophotometer. The enzyme activity was estimated depended on the degradation of casein protein to small peptides and soluble amino acids, the absorbance was measured at 280 nm since one unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01in one minute under experimental conditions.

2.4.3 Determination of protein concentration

It was determined due to Bradford, 1976.

2.5 Mutagenesis of Pseudomonas aeruginosa

Selected *P. aeruginosa* isolates was subjected to mutagenesis using a physical and chemical mutagen in order to obtain a mutants characterized with their higher ability for protease production in comparison with the productivity of wild-type isolate.

2.5.1 Physical Mutagenesis of Pseudomonas aeruginosa by UV radiation

Mutagenesis by UV irradiation was done according to David, (2005) by subjected a fresh culture of *P. aeruginosa* to UV radiation in a dark place using the UV- transilluminator. Chamber of irradiation (approximately15×25 cm) exposed sample in a 2glass Petri dish to UV ray with 2.5 J/m², the distance between UV source and irradiated suspension was 11 cm. *P. aeruginosa* was cultivated in a 10ml nutrient broth at 30°C for 18hrs, followed by centrifuging at 4000 rpm for10min.

The cell pellets were diluted with a sterile phosphate buffer (pH 7), and adjusted to a concentration of 10^6 CFU/ml, then 0.1ml of the cell suspension was poured in a sterilized Petri dishes containing nutrient agar and exposed to UV ray at a wave length of 254nm for different durations (10, 20,30, 40,50, 60,70,80 and90 seconds respectively). After each treatment the bacteria was streaked on nutrient agar plates and incubated over night at 37°C to determine the viable cell count and survivals of *P. aeruginosa*. Bacterial cells subjected to the dose at which 90% of the cells were killed (LD90) were considered as mutants and plated on skim milk agar, then used for further study.

2.5.2 Chemical Mutagenesis of *Pseudomonas aeruginosa* by NTG (1-methyl-3-nitro-1-nitroso-guanidine)

Mutagenesis by NTG was done according to Borwring and Morris, (1985) and as following:

P. aeruginosa was cultivated in nutrient broth at 37°C for 18hrs, then bacterial cells were centrifuged at 4000 rpm for 10 min. The cell pellets were diluted in a sterile phosphate buffer (pH 7.0), and adjusted to a concentration of 10^6CFU/ml, then 1ml of the cell suspension was incubated with 1 ml of 0.1 mg/ml NTG solution at 37°C for different periods of time (0,20, 40,60,80,100 120) min, 0.1 ml serially diluted aliquots were taken after each treatment and plated on nutrient medium agar plates and incubated at 37°C for overnight to determine the viable count and survivals of *P. aeruginosa.* Bacterial cells subjected to the dose at which 90% of the cells were killed (LD90) were considered as mutants and plated on skim milk agar, then used for further study.

2.5.3 Selection of mutants

Mutants a raised after physical mutagenesis were selected and propagated in nutrient broth for 16 hours at 37C°, then 0.2ml from culture was used to inoculated the production medium and enzyme activity was measured.

3 Result and Discusion

3.1 Isolation and Identification of *Pseudomonas aeruginosa*

A total of 150 clinical sample were collected from patients suffering from burns, wounds, otitis media and UTIs infection in four hospitals during the period from 1/7/2017 to 1/12/2017. Results showed that among the total of 150 clinical samples that were collected from different cases of (burns, otitis media and UTI) infection. only 92 isolates (61.3%) were gave typical morphological characteristics and biochemical test that related to *Pseudomonas aeruginosa* while the rest 58 isolates (38.7%) may belong to other pathogenic bacteria from different genera as shown in table (1).

TABLE 1

Source of isolation of *Pseudomonas aeruginosa* from different clinical cases.

Source ofsample	No. of samples	No. of positive strains	Percentage %
Burns	42	37	40.21
Wounds	37	26	28.26
Otitis media	51	21	22.82
UTI	20	8	8.7
Total	150	92	100

The isolation rate of *p. aeruginosa* from burns was high (40.21%) while the rate of isolation of bacteria from other clinical cases such as wound, otitis media and UTIs were (28.26%, 22.82%, 8.7%) respectively. Results of isolation in this study has been agreed with Gamal *et al.,* (2007) who collected out of (145) clinical specimen from three hospitals in Minia, Egypt. 107 strains (74%) were belong to *P. aeruginosa spp. Pseudomonas aeruginosa* is the fourth most commonly-isolated nosocomial pathogen accounting for (10.1 %) of all hospital-acquired infections (Kenneth,2011).

3.1.1 Microscopic examination

Identification of the suspected isolates by gram stain is depend upon morphological characteristics (size, shape, arrangement). All strains were appearing as gram-negative rods, single or pairs, slender or oval shaped organisms, short to medium length, straight or slightly curved, non-sporulating, motile gram-negative rods that grows aerobically that agreed with (Van, 2003; Fuchs *et al*., 2001).

3.1.2 Biochemical tests

According to the results of morphological and microscopic characteristics, bacterial isolates (92) were subjected first to number of biochemical tests, then further identified by Api 20E. Pseudomonas aeruginosa were gave positive results in oxidase test, catalase test. Glucose was oxidized in oxidation / Fermentation (O/ F) test with absence of gas formation from

glucose. Garrity et al., (2005) revealed that P. aeruginosa gave a negative result in gas production and H2S precipitation. Citrate is utilized as source of energy in the citrate utilization test. Its gave K/K profile When grown on TSI (total suger iron) , meaning that the medium will not change color and there is no fermentation of sugars. P. aeruginosa isolates were giving negative results in indole production test, methyl red-voges proskauer tests, urease test as shown in table 2.

Table 2

cultural characteristics and Biochemical tests of Pseudomonas aeruginosa isolates.

Biochemical test for p. aeruginosa	Result
Grape-like smell	+
Catalase	+
Oxidase	+
Triple sugar iron fermentation	k/k
Gas production	-
H2S production	-
Citrate utilization	+
Pyocyanin on King A	+
Fluorescein on King B	+
Growth on cetrimide	+
β-hemolysis on blood ag	+
Lactose fermentation	-

3.1.3 API 20 E system

In API 20E system, Pseudomonas aeruginosa isolates were subjected to further identifications. It's give positive results in Arginine Dihydrolase (ADH), Citrate utilization (CIT), Arabinose fermentation (ARA) but it gives variable results in Gelatin liquefaction (GEL) as shown in figure 1**.**

Fig. 1. Results of identification of *Pseudomonas aeruginosa* isolates by API 20 E system

P. aeruginosa Isolates were give negative results in B-lactamase test (ONPG), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), H_2S production, urease production (URE), Indole production (IND), Tryptophane deaminase (TDA), Voges Proskauer (VP), mannitol, inositol, Sorbitol, rhaminose, sucrose, melibiose, amygdaline and Glucose Fermentation. The result of API 20 E system reveal that only 92 strains from total of 150 isolates were identified as *Pseudomonas aeruginosa*. A study by Maina *et al.* (2014) deducted that Api20E system is a robust bacterial identification method for human infections which can serve small clinical microbiology laboratories that may not be afforded by automated systems.

3.1.4 Identification of bacterial isolates by VITEK 2 system

Pseudomonas aeruginosa isolates Identified by VITEK 2 system, by using the GN (Gram Negative) card. Only 10 bacterial isolates that identified first by certain biochemical test and Api20E were further identified by VITEK 2 system. The VITEK 2 system is an easy-to-handle system that provides a rapid 4 to 15 hrs. and reasonably accurate means for the identification of most commonly isolated species and accurately detects.

Joyanes *et al.*,(2001) tested routinely 146 isolated strains of non-fermenting gram-negative rods by VITEK 2 system and ID-GNB cards with 91.6% similarity in identification . Ineset et al., (2009) founded that the correct identification rates of P. aeruginosa were 90.1% by using VITEK 2 identification card. Garcia-Garrote et al., (2000) declared that VITEK 2 system is a simple, accurate and rapid method. Also its gives a good impact on the work in the laboratories of clinical microbiology.

3.2 Screening for the ability of local *P. aeruginosa* isolates in protease Production

The ability of the locally isolates of *P. aeruginosa* in protease production was screened to select the most efficient isolate in protease production and as follow:

3.3.1 Semi- quantitative(qualitative) screening for protease production

Semi- quantitative screenings for protease production by locally isolate of *p. aeruginosa was* used for detecting the ability of these isolates in grown on skim milk agar medium .From the total of 92 isolates that gave typical and morphological characteristic and biochemical test related to *p. aeruginosa*, only 36 isolates were used in screening for protease production skim milk agar as shown in table (3).

Results showed that all thirty-six isolates were able to grow and hydrolyze skim milk agar medium forming a halos zone of hydrolysis around the colonies with a variable degree, as showed in figure (2).

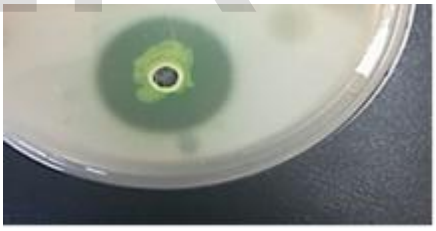

Fig. 2. Proteolytic activity of *p. aeruginosa (*P3) isolate on skim milk agar after incubation at 37°C for 24hrs.

Results mentioned in table (3) showed that the ratios of hydrolysis zone were ranged between (5-28) mm for different isolates. Among them, *P3* isolate was the most efficient one in protease production that gave the highest hydrolysis zone (28mm) on skim milk agar medium.

Table 3

ability of locally isolates of *Pseudomonas aeruginosa* in protease production.

No. *Pseudomonas aeruginosa* Isolates	Diameter of hydrolyzed zone (mm)	No. *Pseudomonas aeruginosa* Isolates	Diameter of hydrolyzed zone (mm)
p1	21	P19	15
P2	18	P20	17
P3	28	P21	11
P4	12	P22	13
P5	9	P23	6
P6	7	P24	18
P7	12	P25	9
P8	15	P26	16
P9	10	P27	15
P10	14	P28	12
P11	8	P29	20
P12	13	P30	8
P13	6	P31	7
P14	9	P32	9
P15	5	P33	17
P16	10	P34	8
P17	21	P35	6
P18	13	P36	9

Bayoudh, *et al.,* (2000) noticed that Protease production from *P. aeruginosa* isolates were selected by growth on skim milk agar. Bacteria showing maximum zones of clearance around their colonies were selected (<20) mm.

3.2.2Quantitative screening

Quantitative screening for ability of locally of p. aeruginosa isolates in protease production was achieved by determining of enzyme specific activity in culture filtrate of these isolates after culturing in broth production medium at 37C°for 24 hrs Result indicated in table (3-4) showed that all of *P. aeruginosa* isolates were protease producer with a variable degrees. Specific activity in culture filtrate of these isolates were ranged between 0.02 and 0.123 U/mg.

Among these isolates *p. aeruginosa* p3 isolates from UTIs patient was the most efficient one in protease production because the specific activity of protease in crude filtrate of this isolate was 0.123U/mg. According to these result *pseudomonas* P3 isolate was selected for further studies on protease production after mutagenesis by physical and chemical methods The differences in the ability of the isolates in protease production are due to genetic variations of the genes responsible for the production of protease (Whealer *et al.,* 1991).

Maies, 2013 reported that all isolates of *Aeromonas* isolates able to produce protease with variable degree and selected the more efficient one to be used for improving its ability in protease production by mutagenesis.

TABLE 4

Specific activity of protease in culture filtrate of locally isolates p. aeruginosa after incubation at 37C° .

Isolates	Specific activity U/mg	Isolates	Specific activity U/mg
P1	0.0901	P12	0.053
P2	0.0833	P13	0.043
P3	0.123	P14	0.046
P4	0.0432	P15	0.0421
P5	0.021	P16	0.081
P6	0.03	P17	0.0885
P7	0.072	P18	0.0545
P8	0.083	P19	0.053
P9	0.08	P20	0.061
P10	0.075	P21	0.047
P11	0.044	P22	0.026
P23	0.043	P30	0.0845
P24	0.76	P31	0.052
P25	0.042	P32	0.055
P26	0.078	P33	0.046
P27	0.051	P34	0.0448
P28	0.062	P35	0.041
P29	0.0755	P36	0.0466

3.3 Mutagenesis of *p. aeruginosa* P3 isolates

In order to enhance the ability of locally isolated p. aeruginosa P3 in protease production by random mutagenesis, this isolate was subjected to physical and chemical mutagens and as follows:

3.3.1 Physical mutagenesis

Since several publications have shown that there are variations in UV stimulation for enhance protease production (Cockell *et al.*, 2000). Physical mutagenesis was achieved by subject the cell suspension of *p. aeruginosa P*3 isolate to UV ray at a wave length of 254 nm 2.5 J/m², for different durations (0,10, 20, 30, 40, 50, 60, 70, 80 and 90) seconds respectively.

Results indicated in figure (3-3) showed that the LD90 was reached after 70 seconds of irradiation by UV-ray and all viability of *p. aeruginosa P*3 isolates was lost after 90 seconds of irradiation. Survivals of irradiated bacterial cells obtained after subjection to LD90 of UV- ray were selected and screened according to their ability to produce protease.

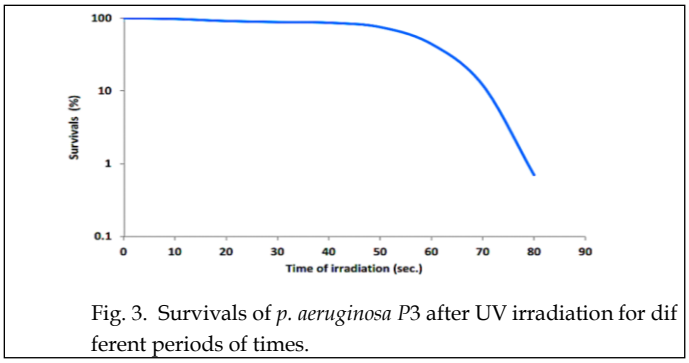

Fig. 3. Survivals of *p. aeruginosa P*3 after UV irradiation for dif ferent periods of times.

Results revealed that the most efficient mutant isolate in protease production was P3 when its specific activity of protease

in culture filtrate reached 0.431 U/mg compared with 0.123 U/mg for the wild type. *Pseudomonas aeruginosa* isolated from wound infection were mutated by UV light and examined for production of protease enzyme result revealed that protease specific activity reached to 0.65 U/mg of protease as compared to the wild type, which yielded 0.34 U/mg. This result showed an increase in protease production by 0.31 fold after mutation by UV light (Gohel *et al*., 2004).

3.3.2 Chemical mutagenesis by NTG

Chemical mutagenesis was achieved by using by 1-methyl-3-nitro-1-nitroso-guanidine (NTG) to induce random mutations in the double stranded DNA helix. To generate mutants with higher protease production. For this purpose fresh culture of *p. aeruginosa P*3 was incubated with by (NTG) in a concentration of 0.1mg/ml at 30°C for different periods of time. Results illustrated in figure (3-4) showed that survival of the bacterial cells was decreased with increasing time of incubation of the mutagen till complete death after 120 minute of incubation, while the LD90 was reached after 80 min. Aliquots of cell culture were taken after this time of incubation and enzyme specific activity was measured to evaluate the overproducer protease production. Results revealed that enzyme specific activity of LD_{90} of *p. aeruginosa* p3 isolate after 80 min. was 0.0894 U/mg.

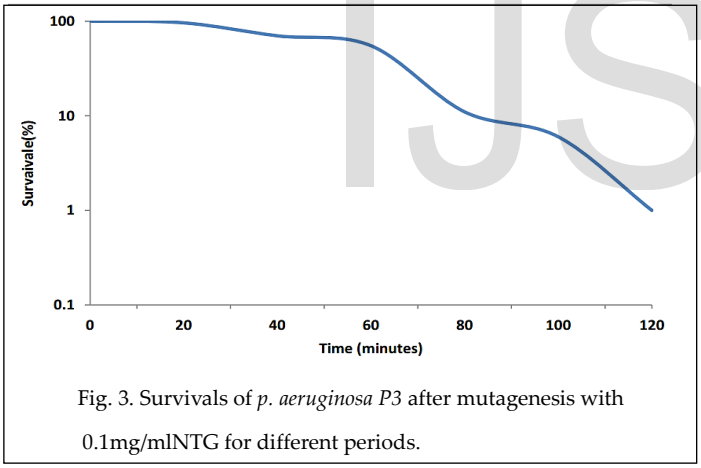

Fig. 3. Survivals of *p. aeruginosa P3* after mutagenesis with 0.1mg/mlNTG for different periods.

According to these results it could be concluded that physical mutagenesis using UV irradiation was more efficient than the chemical mutagenesis by NTG to enhance the ability of locally isolated *p. aeruginosa* isolate in protease production, highest specific activity of protease produced by this mutant (P3) isolate was 0.431 U/mg after UV radiation in comparison with 0.0894 U/mg for the mutant obtained after mutagenesis by NTG , so that the over producer mutant *p. aeruginosa* P3 isolate was selected and used for determining the optimum conditions for protease production. This result has been agreed with Xiu-Gong Gao *et al*., 2000 who reported that *pseudomonas aeruginosa* protease activity have been increased by UV mutation more than mutation by chemical mutation such as NTC.

It is well known that the most efficient *p. aeruginosa* mutants were raised after reaching the LD90 as described by Borwring and Morris, 1985. Results showed that many mutants were obtained after 80 sec. of exposure to UV.

This result has been agreed with Xiu-Gong Gao *et al*., 2000 who reported that *pseudomonas aeruginosa* protease activity have been increased by UV mutation more than mutation by chemical mutation such as NTC.

4 Conclusion

1-Isolates of Pseudomonas aeruginosa isolated from clinical samples of different age patients was identified by certain biochemical tests.

2- 92 psedomonas aeruginosa isolates were identified by Epi 20 E system then ten isolates of them was identified by VITEK 2 seystem.

3- All Locally *P. aeruginosa* isolated from different clinical cases are able to produced protease enzyme and among them *P. aeruginosa P3* isolate was most efficient one in protease production, favored its use in the future for the industrial applications.

3- Mutagenesis of *P. aeruginosa P3* isolate by UV - ray was better than mutation by chemical mutagenesis such as NTG for obtaining protease overproducer mutants.

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4 Conclusion

Although a conclusion may review the main points of the paper, do not replicate the abstract as the conclusion. A conclusion might elaborate on the importance of the work or suggest applications and extensions. Authors are strongly encouraged not to call out multiple figures or tables in the conclusion—these should be referenced in the body of the paper.

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