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Optimizing arginine deiminase production from *Pseudomonas aeruginosa* clinical isolate

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Abstract:

This study was aimed to isolation, identification of *Pseudomonas aeruginosa* then optimizing its arginine deiminase production conditions. This bacterium is a gram negative rod shaped isolated from burns, wounds and urinary tract infections, identification depending on morphological, cultural and biochemical tests, confirmed by VITEK –II system showed that 13 out of 59 bacterial isolates belong to *P. aeruginosa* characteristics. Arginie deiminase activity was screened in these isolates calorimetrically by estimating the level of citrulline and the results showed that these isolates were arginine deiminase producers with variable degrees. It has been found that *P. aeruginosa* P3 had the highest specific activity of 0.42 U/mg protein. The effect of culture medium components on ADI production was estimated and the maximum production was achieved following supplementation of the production medium with 0. 5% maltose, 0.5% Trytone, 50mM arginine, pH 7.5, inoculated with a 10^8 cells/ml of fresh bacterial culture and incubated at 37C^o for 24h.

Keywords: clinical isolate ; arginine deiminase production ; and Pseudomonas aeruginosa;

INTRODUCTION

There are approximately 340,000 genera of bacteria in the world, many of them indigenous in the environment. Pseudomonds are a wide spread genera, comprising of many species, some of which are also important opportunistic pathogens humans and animals.Species of pseudomonads include, amongst many others, *P. aeruginosa*, *Pseudomonas fluorescens and Pseudomonas stutzeri*(1).

The genus *Pseudomonas* was described by Migula in 1894 according to the morphological characteristics of its members. However in 1984, the genus was revised, and a subdivision of five groups was implemented based on the DNA–DNA hybridisation (DDH) and rRNA-DNA hybridisation results(2)..

P. aeruginosa may cause multiple infections in man that vary from local to systemic and from benign to life threatening. During the last few decades, the cosmopolitan Gram-negative bacterium has become one of the most frequent causative agents of nosocomial infections associated with substantial morbidity and mortality (3). Also, Murphy *et al*., (4) stated that chronic airway infections with *P. aeruginosa* are a major cause of morbidity in people with cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD). The opportunistic infections of *P. aeruginosa* is multifactorial, as suggested by the large number of cell-associated and extracellular virulence determinants; some of these determinants help colonization, whereas others facilitate bacterial invasion. Arginine metabolism via the ADI pathway is well established to be broadly present in a number of microorganisms and enables them to adapt to hostile environmental niches and host defenses (5,6). Growth and enzyme production of any organism are greatly influenced by both environmental conditions as well as the nutrients available in the growth medium. Carbon was one of the major elements in the medium composition for the metabolic activities of the isolate (7). In the present study,we attempt to optimize production medium using one factor at a time approach for the enhanced production of ADI obtained from *P. aeruginosa.*

MATERIAL AND METHODS

Specimens collection

A total of 75 specimens were collected from burns, wounds and urinary tract infections from Al-yarmok hospital in Baghdad. Swab specimens were aseptically transferred under cooling conditions to the laboratory for analysis.

Isolation of *Pseudomonas aeruginosa*

According to (8), swabs were taken carefully from the site of infection and placed in tubes containing transferred medium to maintain the swab wet during transferring to laboratory.

Cultural examination

Morphological characteristics of colonies were studied on cetramide agar .Color; size and edge of the colonies were recorded after 24 hours of incubation at 37˚C.

Microscopic examination

A single colony of each isolate was fixed on a clean slide to study gram stain, under light microscope.

Biochemical tests

The suspected isolates were subjected to the biochemical tests as mentioned by (8), and as follow:

A Catalase test

A single colony of each bacterial isolate was taken and smeared on clean glass slide , then a drop of hydrogen peroxide, 3drops was flooded from 1.0 ml of 3% hydrogen peroxide.Presence of gaseous bubbles indicate a positive result.

B Oxidase test

This test was done using filter paper moistened with few drops of afreshly prepared solution of tetramethyl-ρ-phenylenediaminedihydrochloride. Aseptically, a clump of cells was picked up from the slant growth with a sterile wooden stick and smeared on the moistened paper. Development of a violet or purple color within 10 seconds indicates a positive result.

C- Indole test

Colony was inoculated into peptone water broth and incubated at 37°C for 24 hrs in a shaker incubator. After incubation,few drops of Kovac's reagent were added. Presence of pink colored ring indicates a positive result.

D- Citrate utilization test

A loopful of colony was streaked onto a simmon citrate agar slant, and then incubated for 24 to 48 hrs at 37°C in incubator. Change in medium color to blue color indicates apositive results.

Identification of suspected bacteria by VITEK 2 system

The VITEK 2 is an automated microbiology system utilizing growth-based technology. Used for bacterial identification.

Screening the ability of *P. aeruginosa* for arginine deiminase production.

A- Semi quantitative screening

Each local isolate of *P.aeruginosa* streaked on nutrient agar medium and incubated at 37 °C for 24h. A single colony was then taken and placed on the center of M-9 medium plate. The plate was incubated at 37°C for 48h. Ability of *P.aeruginosa*in arginine deiminase production was measured based on the presence of pink zone around each the colony.

B- Quantitative screening

ADI activity was estimated after drawing the standard curve of citrulline in the reaction mixture (1ml) containing 0.4ml of 50mM phosphate buffer0.4 ml L-arginine and 0.2 ml cell suspension incubated at 37ºC for 15min., the reaction was stopped by the addition of 10% trichloroacetic acid, then centrifuged at 6000 rpm for 30 min. The citrulline formed was measured by the modified Archibald method (9), 1.4ml of acid mixture (96% H_2SO_4-85% H_3P0_4; 1:3,vol/vol) was added to 1 ml of the supernatent after centrifugation of trichloroacetic acid-treated sample, and 0.5 ml of 3% diacetylmonoxime were added, mixed together, then boiled in the dark for 15min, cooled in the dark for 10 min, the absorbance was measured at 490 nm and the enzyme activity was estimated according to the standard curve of citrulline.

Protein concentration was determined according to the method described by Bradford .

A Optimum Carbon Source

Various carbon sources (glucose, galactose, starch, lactose, and maltose) were added, individually, to the minimal medium. At a final concentration of 0.5 %.

B Optimum Nitrogen Source

Various nitrogen sources (peptone, casein, tryptone, yeast extract and gelatin) were added individually to the production medium.

C-Effect of arginine concentration

Production medium containing different arginine concentrations (20, 30, 40 ,50 and 60mM) was inoculated with the selected isolate. And grown at optimum conditions.

D- Optimum pH on enzyme activity

The production medium was prepared at different pH values (5-9) , then bacterial isolate was inoculated,incubated at 37Cº for 24h .

E- Optimum temperature for enzyme activity

The bacterial isolate was inoculated in production culture medium of bacterial culture and incubated at different temperatures (25, 30, 35, 40, 45, 50C^0) for 24h.

F- Optimum Inoculum Size

The best incolum size was achieved by inoculating the production medium, individually with an inoculum size of (10^3,10^4,10^5,10^6,10^7,$10^8$and 10^9CFU).

RESULTS AND DISCUSSION

Isolation and identification of bacterial isolates

Cultural characteristic

These samples were cultured on to MacConky agar primarily identified according to their cultural and morphological characteristics. Only 59 of them able to grow on MacConky agar, colonies were pale yellow in color, non-lactose fermenter. MacConky agar know to contain bile salts and crystal violet which promot the growth of Enteroacteriaceae and gram negative rods, also lactose in this medium differentiate between lactose fermenting and non-lactose fermenting bacteria (10,11). When these isolates grown on to nutrient agar,greenish coloration was observed due to production of Pyoverdin pigment. While when cultured on cetrimide agar, thirteen isolates were able to grow with grape-like shape.These features come in accordance with the corresponding cultural characteristics of *P. aeruginosa* as mentioned by Jebur *et al.,* (2015).

Microscopic characterization

The suspected bacterial isolates were negative in gram staining. In addition, the cells were mainly single non-spore forming small rods. This result agreed with described by(13) for *P.aeruginosa* characteristics.

Biochemical characterization

Depending on their efficiency in producing pyocyanin, the suspected *Pseudomonas* isolates were subjected to the related biochemical tests. 13 isolates were positive for catalase production, oxidase production, gelatin liquefaction, protease production, pyocyanin production on King A and production of fluorescent on King B, as well as able to grow at 42ºC. Adversely, these isolates were negative for gas production and H_2S production.

Depending on the cultural characterization and results of biochemical tests, these 13 isolates were found to be belonging to *P. aeruginosa*. (14) revealed that such characteristics usually are coming in agreement with those fitting to *P. aeruginosa.* Also, (15)stated that growth at 42ºC is a characteristic feature of *P. aeruginosa*, since most other *Pseudomonads* are unable to grow at 42°C.

Identification of bacterial species by VITEK system

To confirm the result of biochemical test, Vitek 2 system with GN (Gram-negative) cards was applied for 13 suspected isolates. The results of VITEK 2 system founded that all isolates were 99% similar to the characteristics *of P. aeruginosa*.

In a similar study, (16) tested forty two isolates, 25 isolates was identified as *P. aeruginosa* using Vitek 2system. The correct identification rates *of P. aeruginosa* using this automated technique were 90.7%. Also, (17) founded that the correct identification with 100% probability, by calorimetric Vitek 2 system which used as a recent aid for more confirmation for identification of *P. aeruginosa*.

Semi qualitative screening

Semi- quantitative screening for arginine deiminase production was achieved by detecting the ability to form a pink zone around each colony when grown on M-9 medium containing phenol red. Results showed that the pink color directly proportional to the quantity of enzyme produced. However, among the thirteen bacterial isolates, *P. aeruginosa* P3can be considered as the highest arginine deiminase producer which gave clear pink zone when compared to the other isolates. During ADI production, the formation of pink zone around the colonies due to production of ammonia, which is the end product of arginine hydrolysis indicated that there was no ammonia formation in control plates. This revealed that change incolor of the medium (red or dark orange to pink) around the colonies due to ADI production (18,19,20,21).

Optimum carbon source In this study, the maximum specific activity of ADI from *P.aeruginosa* P3 was obtained when the production medium was supplemented with maltose as a sole source of carbon and energy gave higher rate of ADI productivity (1.2 U/mg protein), followed by lactose, galactose, glucose and starch, respectively (Fig.1). The maximum production of ADI was achieved when a combination of arginine and maltose was incorporated in the medium. However, reverse result was seen when glucose was added to the arginine medium. Glucose- arginine decrease the activity of the arginine fermentation system which is comparable with the results obtained earlier for *Enterococcus faecium* by (22). In another study the presence of glucose decreased the enzyme yield to half when compared with galactose, which may be due to glucose catabolic repressive nature (23).

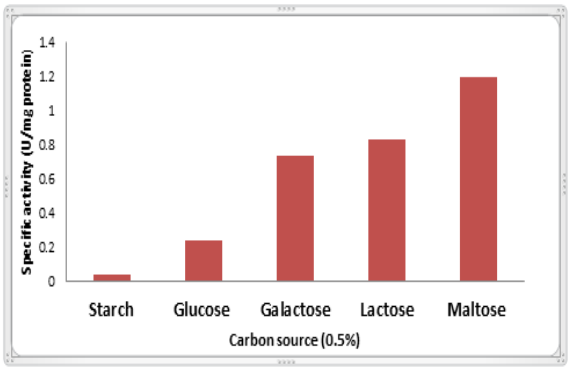


Figure (1)Optimum carbon source for arginine deiminase produced by *P.aeruginosa* P.3 after incubation at 37°C for 24 hr .

Optimum nitrogen source

Nitrogen source is another major nutrient after carbon that is essential for the growth of microorganisms in larger amount. Nitrogen is an essential part in protein, nucleotides, enzymes and cofactor which play vital role in the metabolism (23).

Among different nitrogen sources (peptone, tryptone, yeast extract, casein and gelatin), tryptone proved to be the best for ADI production from *P.aeruginosa* with specific activity reached to 3 U/mg protein (Fig.2). (25) reported that the optimum arginine deiminase production by *Enterococcus faecium* was observed with treptone.

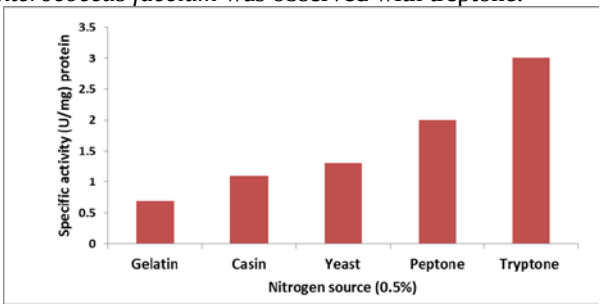
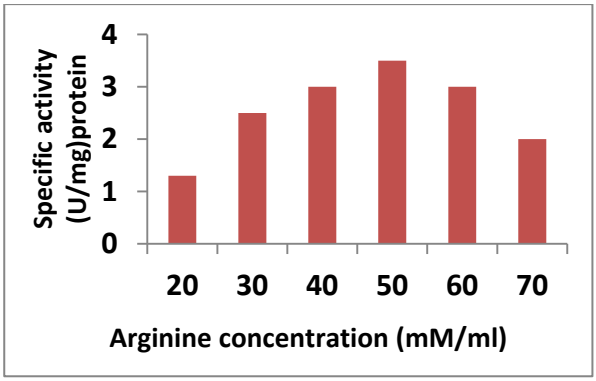


Figure (2) Optimum nitrogen source for arginine deiminase production by *P. aeruginosa* P3 incubated at 37°C for 24 hr

Optimum arginine concentration

Demonstrates the effect of these concentrations on the production of enzyme, 50mM arginine was the most suitable concentration for enzyme production,at this concentration the specific activity reached to 3.5U/mg protein (Fig.3). *(*26) recorded that the optimum concentration of arginine in *E.faecalisas* culture medium was50mM/ml .



(Figure3):Effect of arginine concentration on arginine deiminase production by *P.aeruginosa* after incubation at 37°C for 24 hr.

Optimum pH for arginine deiminase production

Arginine deiminase production was examined at PH values ranged between 5 to 9, maximum arginine deiminase production was obtained when the pH value of the production medium was adjusted to 7.5, at this value the enzyme specific activity in culture filtrate was 4 U/mg protein (Fig.4). The results explained that the enzyme is produced in a wide range of pH values, but it favored pH 7.5, this may be attributed to the fact that this bacteria was isolated from the human body which has a neutral pH. The pH of production media affected the enzyme activity by influence the solubility of nutrients and transition of them through the cell membrane and its effect on: ionic state of the substrates, stability of enzymes, interaction with the growth of bacteria and production of enzymes (27). Since, changes in the pH or acidity of the environment can take place that would alter or totally inhibit the enzyme from catalyzing a reaction. This change in the pH will affect the polar and non-polar intramolecular attractive and repulsive

forces and alter the shape of the enzyme and the active site as well to the point where the substrate molecule could no longer fit, and the chemical change would be inhibited from taking place as efficiently or not at all (28). Our result is in agreement with (29) who noticed that the maximum production of arginine deiminase from *Enterococcus faecium M1*was obtained when the production media was adjusted to pH 7.5.

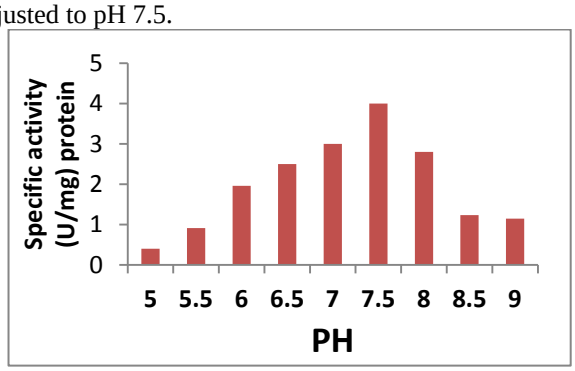
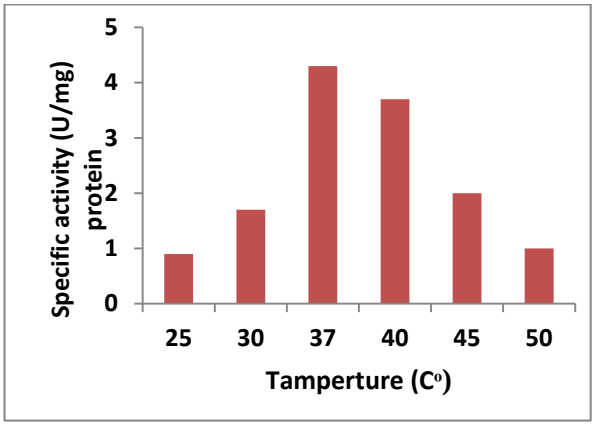


Figure (4) Optimum PH for arginine deiminase produced by locally *P.aeruginosa* after incubation at 37 °C for 24hr

Optimum incubation temperature

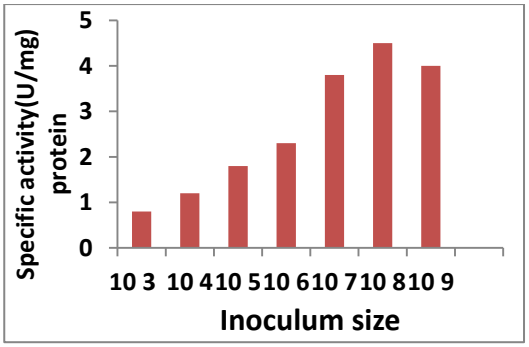
Different incubation temperatures (25-50ºC) were tested to determine the optimum one for ADI production by *P.aeruginosa* P3. Maximum production of ADI was reached at 37ºC,the specific activity was 4.3 U/mg protein at this temperature (Fig.5). However, other temperatures led to decrease the enzyme activity. These results could be attributed to the fact that this isolate favored this temperature for growth and metabolism. Furthermore, *P.aeruginosa* tolerate higher temperatures and can grow and produce the enzyme at 50ºC but with lower production than 37ºC.



(Figure5): Effect of incubation temperature on arginine deiminase production by P.aeruginosa P3

Optimum Inoculums size

arginine deiminase production was increased slightly with increasing the inoculum size with maximum production at 1×10^8 CFU/ml, and specific activity reached to (4.5 U/mg protein) show (Fig. 6).. In a previous study submitted by (30) maximum ADI production was obtained at 1×10^8 CFU/ml with specific activity reached at 0.46 U/mg.



(Figure.6)Optimum inoculum size for arginine deiminase production by P.aeruginosa after incubation at 37°C for 24 hr

CONCLUSIONS

*P. aeruginosa*is P3 local isolate can be used as an efficient source for arginine deiminase production.

Nutritional and environmental conditions for ADI produced by *P. aeruginosa*is P3 isolate was optimized when the minimal salt medium supplemented 0.5% maltose a carbon source, 0.5%with a tryptone as a nitrogen source, 50mM arginine,adjusted at pH 7, inoculated with 10^8 CFU/ml. and incubated at 37 °C.

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