

## ORIGINAL RESEARCH ARTICLE

**Isolation, Characterization and Purification of Exogenous Protein from *Pseudomonas aeruginosa*****K. Viswanathan\***

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**ABSTRACT**

*Pseudomonas aeruginosa* is measuring 0.5 to 0.8  $\mu\text{m}$  by 1.5 to 3.0  $\mu\text{m}$  almost all strains are motile by means of a single polar flagellum. The bacterium is ubiquitous in soil and water and on surface in contact with soil or water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of  $\text{O}_2$  if  $\text{NO}_3$  is available as a respiratory electron acceptor. The typical *Pseudomonas* bacterium in nature might be found as a biofilm, attached to some surface or substrate, or in as a planktonic form as a unicellular organism, actively swimming by means of its flagellum, *Pseudomonas aeruginosa* is one of the most vigorous, fast swimming bacteria seen in hay infusions and pond water samples. *Pseudomonas aeruginosa* has very simple nutritional requirements it is often observed growing in “distilled water”, which is evidence of its minimal nutritional requirements, in the laboratory, the simplest medium for growth of *Pseudomonas aeruginosa* consists of acetate as source of carbon and ammonium sulphate as a source of nitrogen. The bacterium is isolated and characterized and the exogenous protein isolation is carried out in spent media of overnight to 8 hour culture broth of *Pseudomonas aeruginosa*. The presence of protein is confirmed by Ninhydrin test and APS test both give their characteristic positive results. The protein concentration of the spent media is estimated by popular Lowry’s method and found to be 19.98 mg of protein present in one ml of the spent media. The SDS – PAGE done with molecular marker of ovalbumin (43 kDa) in 11% gel concentration yield only a single band in the test sample indicates that only one type of protein is present in the sample. Which was further resolved and its molecular weight is found to be 55 kDa.

**Key words:** *Pseudomonas aeruginosa*, Protein, Ninhydrin test, Lowry’s method and SDS-PAGE.**1. INTRODUCTION**

*Pseudomonas aeruginosa* is a free – living bacterium, found in soil and water, occasionally on the surface of plants and rarely on the surface of animals. Members of the genus are well known to plant micro biologists because they are one of the few groups of bacteria that are true pathogens of plants. In fact, *Pseudomonas aeruginosa* is occasionally a pathogen of plants however, *Pseudomonas aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its occurrence as a nosocomial pathogen and studies indicate that antibiotic resistance is increasing in clinical isolates.

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bacterium is ubiquitous in soil and water and on surface in contact with soil or water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of  $\text{O}_2$  if  $\text{NO}_3$  is available as a respiratory electron acceptor. The bacteria *Pseudomonas* in nature might be found as a biofilm, attached to some surface or substrate, or in as a planktonic form as a unicellular organism, actively swimming by means of its flagellum, In its natural habitat *Pseudomonas aeruginosa* is not particularly distinctive as a *pseudomonades* but it does have a combination of physiological traits that are note worthy and may relate to its pathogenesis.

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laboratory, the simplest medium for growth of *Pseudomonas aeruginosa* consists of acetate as source of carbon and ammonium sulphate as a source of nitrogen. Its optimum temperature for growth is 37° C, and also it can be able to grow at temperatures as high as 42° C [2]. It is tolerant to a wide variety of physical conditions including temperature; it is resistant to high concentrations of salt and dyes, weak antiseptic and many commonly used antibiotics.

*Pseudomonas aeruginosa* has a predilection for growth in most environments, which is probably a reflection of its natural existence in soil and water. *P. aeruginosa* isolates may produce three colony types natural isolates from soil or water typically produce a small, rough colony, clinical samples, yield a large fried-egg appearance, smooth colony types.

*P. aeruginosa* obtained from respiratory and urinary tract secretions, has a mucoid appearance, which is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play a role in colonization and virulence. *P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment pyoverdine and the blue pigment pyocyanin. The latter is produced abundantly in media of low-iron content and functions in iron metabolism content in the bacterium. Pyocyanin (from “pyocyanous”) refers to “blue pus”, which is a characteristic of supportive infections caused by *Pseudomonas aeruginosa*.

Biofilms of *Pseudomonas aeruginosa* can cause chronic opportunistic infections. These kinds of infections are a serious problem for medical care in industrialized societies; especially for immunocompromised patients and the elderly. They often cannot be treated effectively with traditional antibiotic therapy. Biofilms seem to protect these bacteria from adverse environmental factors [3]. *Pseudomonas aeruginosa* can cause nosocomial infections and is considered a model organism for the study of antibiotic-resistant bacteria. Researchers consider it important to learn more about the molecular mechanisms which cause the switch from planktonic growth to a biofilm phenotype and about the role of inter-bacterial communication in treatment-resistant bacteria such as *Pseudomonas aeruginosa*. This should contribute to better clinical management of chronically infected patients, and should lead to the development of new drugs

*P. aeruginosa* is naturally resistant to a large range of antibiotics and may demonstrate additional resistance after unsuccessful treatment, particularly through modification of a porin. It should usually be possible to guide treatment according to laboratory sensitivities, rather than choosing an antibiotic empirically. If antibiotics are started empirically, then every effort should be made to obtain cultures, and the choice of antibiotic used should be reviewed when the culture results are available.

The aim of the present study was to isolate and characterize the *Pseudomonas aeruginosa* from the study sample and to prepare and maintain a pure mother culture stock for further studies on the bacterium in the future. After the successful preparation of mother stock culture it's used for the isolation, purification, and analysis of exogenous protein produced during the log phase of bacterial growth cycle of *Pseudomonas aeruginosa*. We decided to isolate this protein in the scope to facilitate the better understanding of metabolism, environmental adaptation, biochemistry, pathology, and diagnosis of *Pseudomonas aeruginosa*. As it may serve as a good fundamental work for these studies.

## 2. MATERIALS AND METHODS

### COLLECTION AND TRANSPORT OF SOIL SAMPLE:

Various Agricultural Areas were selected and sample was collected. The samples were collected from the following fields in polythene covers.

- (1) Paddy field – Vellore
- (2) Sugarcane field – Arakkonam

The soil sample from the above mentioned agricultural fields were collected in clean different poly ethane covers and the end of the covers was tightly tied with twine. The Sealed soil were transported to the laboratory and processed for further use.

### ISOLATION AND IDENTIFICATION OF *Pseudomonas fluorescens*

Identification of the *Pseudomonas fluorescens* were carried out by the routine bacteriological methods i.e.,

- a) By the colony morphology
- b) Preliminary tests like Gram staining, Capsule staining, Endospore staining, Motility, Catalase and Oxidase.
- c) Plating on selective media.
- d) By performing biochemical tests.

**AMMONIUM PER SULPHATE PRECIPITATION:**

During Ammonium Sulphate precipitation, The salt as to be added small patrons under constant stirring to prevent increase pf high local concentration, ammonium Sulphate was used for precipitation of total protein at 90% saturation or differential precipitation of protein using different saturation's of the salt, 40% saturation was done.

To precipitate the protein form the culture after complete dissolution of the salt, the solution was equilibrated for approximately one day in cold condition to ensure complete precipitation and then the precipitate was collected by centrifugation.

**IDENTIFICATION OF THE AMINO ACID:**

100 mg of the ninhydrin was weighted accurately and was dissolved in 100 ml of ethanol. 100 ml of the extracted sample was taken and 2 ml of the ninhydrin reagent was added to it test tube the solution was kept in boiling water bath for 10 minutes. After a few minutes, the solution turned violet color formation.

**DIALYSIS METHOD:**

The enzyme obtained by ammonium Sulphate precipitation was suspended in a buffer pH 6.8 and dialysis was done. Dialysis is a process, by which small molecules are selectively removed from a sample containing mixture of both small and large molecule. Dialysis was done for the purification of the sample. Dialysis is effectively accomplished using a special type of membrane known as semi permeable membrane. The semi permeable membrane allows the small molecules to pass freely through holding the large molecules inside.

**PRETREATMENT OF DIALYSIS MEMBRANE:**

For the biological work the membrane are pretreated to remove some undesirable impurities such as glycerol, heavy metals, sulphides etc. i.e. associated during manufacturing process. To remove glycerol, heavy metals, sulphur and also inactivate any Croatian that may be present in the dialysis tube, the tubes is cut into pieces of about 5 to 10 cm and placed inside a beacon containing 500 ml of 2% NaHCO<sub>3</sub>, 100 mM EDTA and boiled for twenty minutes, after 20minutes of boiling, the inside and outside of the tubing are washed with distilled water using squeeze bottle. The tubing is again boiled for 10 min in 1 mM EDTA to remove excess sodium carbonate. The inside and outside of the tubing are again washed

thoroughly as above and stoned in 10% ethanol at 4<sup>0</sup> C.

To dialysis a sample, the tubing of required length is taken (use gloves to handle the tubes and both inside and outside of the tubing is rinsed with the distilled water.) One ending of the tubing is tied secured with a thread. Now using the open end, fill the tube with distilled water. Holding the tube securely by the top, the tube is gently squeeze to cheek whether there is any leakage on the membrane or on the closed end. If there is no leak, the water is removed and the sample solution is filled 2/3 using a pipette or a funnel. Then the open is also closed securely by tying a thread. Now the bag is placed in an appropriate buffer solution and a dialyzed for 3 to 4 hours at required temperature. During the time the small mole hills will be removed from the bag. Change the buffer, if necessary after 3 to 4 hours.

**ESTIMATION OF PROTEIN BY LOWRY'S METHOD:**

The protein content was estimated by the Lowry's method <sup>[4]</sup>.

**DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE**

Samples were analyzed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% separating and 4.5% stacking) to check the purity and determine the molecular mass of the purified protein. The electrophoresis apparatus were neatly washed and assembled the glass plates. The stacking and separating gel were prepared using above composition. The gel solution was heated up to the appearance of clear solution. Pour the separating gel from the gap between the glass plates until <sup>3</sup>/<sub>4</sub> is filled and 2 mm layer of distilled water on top of gel. Let leave the gel up to 30 min for polymerization. The stacking gel was poured over the separating gel. Immediately insert a clean Teflon comb into the stacking gel solution and avoid trapping air bubbles. After polymerization comb was removed, the wells are washed with water. Remove the water by inverting the gel. The gel tanks were filled by running gel buffer. Then glass plates were fixed in Electrophoresis apparatus. The protein samples were taken and mixed with three part of SSB. The mix was boiled at 100°C for 3 min. samples were loaded on the wells. Electrophoresis apparatus attached to the electric power supply in 20 mA to the gel. It was run until dye front reaches the bottom gel. The gel plates were removed from the tank using spatula

carefully. Stacking gel was removed completely from separating gel.

### 3. RESULTS AND DISCUSSION

*Pseudomonas aeruginosa* is a Gram negative rod measuring 0.5 to 0.8  $\mu\text{m}$  by 1.5 to 3.0  $\mu\text{m}$  almost all strains are motile by means of a single polar flagellum. It forms large, opaque, and irregular colonies with distinctive musty or earthy colonies. It gives positive result in the Cetrimide (milk agar) test by forming colonies. Found to be beta haemolytic colonies by blood agar test. In the triple sugar iron test it gives alkaline butt and alkaline slant which interprets as the micro organism streaked is capable of producing alkaline and no acid production was observed. In the bio chemical characterization of *Pseudomonas aeruginosa* gives positive result for the following tests catalase, oxidase, methyl red, citrate, urease, gelatinase, nitrate, indicates that the organism is capable of utilizing these nutrients efficiently. but the result is being negative for Indole and Voges proskauer test and one test left undone that is coagulase test because this test not need by our scope of analysis. The O/F test indicates that the organism is oxidative indicates that it need oxygen for growth and it is aerobic. Thus, the bacteria are isolated as pure culture and mother stock culture was inoculated and the bacterium is characterized using the above mentioned tests. Then to isolate and purifying the exogenous protein ,which we inoculate a loop full of bacteria in Nutrient Broth and incubate it for 8 to 12 hours or overnight at 37 $^{\circ}$  C in a shaker incubator with mild shaking.

After the aliquot is centrifuged and cell is pelleted out and the supernatant was collect for the analysis and purification of exogenous protein form *Pseudomonas aeruginosa*. The presence of protein is confirmed my Ninhydrin test and APS test both give their characteristic positive results indicate the presence of protein in turn the production of exogenous protein by *Pseudomonas aeruginosa*.

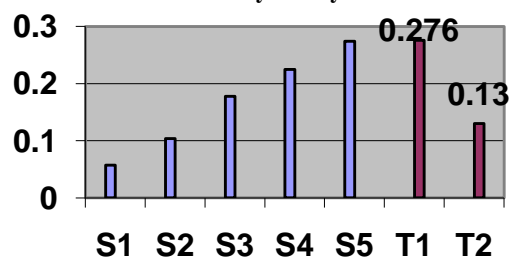
The protein concentration of the spent media is estimated by popular Lowry's method and found to be 19.98 mg of protein present in one ml of the spent media. Then the protein is purified form non proteinous substance by the use of dialysis of the spent media with a suitable buffer and conditions. Now the purified sample is subjected for the isolation and purification of different proteins present in them by SDS -PAGE and molecular weight is determined using Non - Degradative Poly acrylamide Gel Electrophoresis. With % concentration of gel of 5, 7, 9, and 11 % of

acrylamide used with suitable markers. The ferritine marker which is 440 kDa, Catalase molecular marker which is 232 kDa, aldolase is 154 kDa and ovalbumin which is 43 kDa in molecular weight being used. The graph was plotted for various concentration of gel and slope of gel was taken and a separate graph is drawn and molecular weight is calculated to be 55 kDa.

The SDS - PAGE done with molecular marker of ovalbumin in 11% gel concentration yield only a single band in the test sample indicates that only one type of protein is present in the sample, which was further resolved and its molecular weight is found to be 55 kDa. This can be studied further in future with these procedures as a reference card for the analysis of exogenous proteins, or the analysis of exogenous protein from *Pseudomonas aeruginosa* .In which the nature and characteristic feature of the protein may be analyzed.

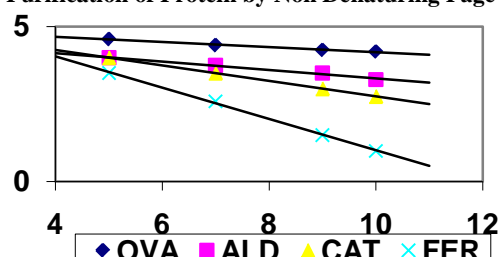
One of our suspicions with protein isolate is that it may be one of the classes of protease produce by the bacterium in the course of its life time. If it is so they can be tested for its possible application in the commercial, industrial and medical scales. The other approach in the future is to isolate proteins and some other bio molecules and various stages of cell cycle at various physiological and bio chemical conditions which helps us understand adaptive nature of the bacterium better and work the molecular mechanics operating behind this. And can be exploited for the future genetic engineering works towards biotic and abiotic stress tolerant GMO's especially the plant varieties. For all these future prospects and researches this study and work will serve as better reference.

Fig 1: Estimation of Protein by Lowry method



X - axis : Volume of working Standard solution(ml)  
Y - axis : O.D(no unit at 620 nm)

Fig 2: Purification of Protein by Non Denaturing Page

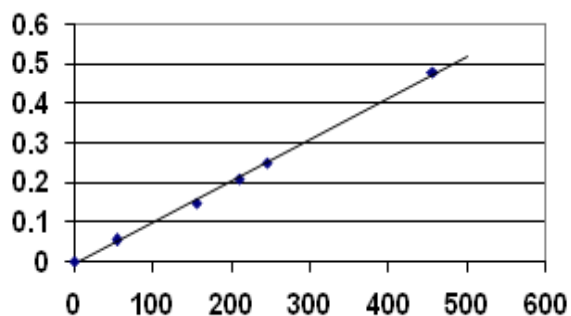


Gel Concentration vs Log of 100 x Rf value

X – axis: Gel concentration in %

Y – axis: Log (Rf x 100) value

Fig 3: Purification of Protein by Non Denaturing Page

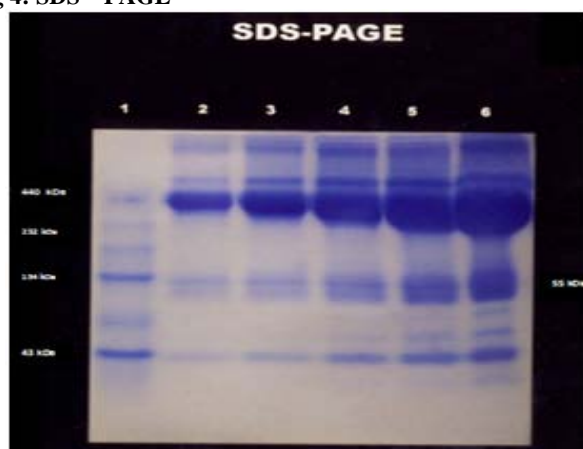


SLOPE Vs MOLECULAR MASSES

X – axis: Molecular mass (kDa).

Y – axis: Slope.

Fig 4: SDS – PAGE



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