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Pseudomonas aeruginosa BUP2—A Novel Strain Isolated from Malabari Goat Produces Type 2 Pyoverdine

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Abstract

This study focuses on the isolation and characterization of a novel strain of siderophore producing bacterium, *i.e.*, *Pseudomonas aeruginosa* BUP2 (*Pa* BUP2) from the rumen of Malabari goat, coupled with qualitative and quantitative analyses of the siderophore produced by it. *Pa* BUP2—a facultative anaerobe was tuned to be an aerobe by repeatedly growing in Benjamin flask. The new isolate was grown in a specially designed semi-synthetic medium, designated as BUP medium, and the yellowish-green pigment produced was identified as a typical siderophore by spectrophotometry, Chromazurol-S assay, thin layer chromatography and isolectric focusing (IEF). The characteristic orange fluorescence upon UV irradiation on chromatogram and absorption maximum at λ_{404} confirmed that the characteristic siderophore produced by *Pa* BUP2 was a typical pyoverdine (PVD). This PVD was further categorized under type 2 by comparing its profile on the IEF gel with that of the representative strains of each PVD types, *viz*., *Pa* O1, *Pa* ATCC 27853 and *Pa*6. Moreover, the type 2 PVD was purified by XAD-4 Amberlite column chromatography and quantified; maximum yield (11.17 mg/ml) was observed on day 4 of incubation (37˚C). Thus, it was confirmed that the bacterium isolated from the rumen content of Malabari goat is a novel strain of *Pa* capable of producing large quantity of PVD type 2 in specially designed BUP medium under aerobic condition, and that its clinical and industrial implications remain elusive.

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Keywords

***Pseudomonas aeruginosa* BUP2, Siderophore, Pyoverdine Type 2, Isoelectric Focusing**

1. Introduction

Fluorescent Pseudomonads represent a heterogenous group of Gram-negative bacteria, extensively studied for the production of yellow-green, water-soluble pigments called bacterial pseuobactins or pyoverdines (PVDs) [1]. Iron is an important element necessary for bacterial growth and survival. Due to its urge to rapidly oxidize to insoluble Fe^{3+} compounds under aerobic conditions in natural environment, iron is not readily accessible to many microorganisms. In order to circumvent this problem, many bacteria secrete iron-chelating molecules called siderophores [2]. PVDs are a group of flourescent siderophores having high affinity for iron and are synthesized under iron-deficient growth environments [3]. They are peptide-derived primary siderophores, composed of three structural parts: an invariant dihydroxyquinoline chromophore conferring color and fluorescence, an acyl moiety and a variable peptide side chain comprising 6 to 12 amino acids, some of them being unusual. PVDs have three iron (III) binding sites; the peptide chain lodges two of these iron binding sites, and the catecholate of the chromophore harbors the third one [4]. The peptide moiety is principally involved in receptor recognition, whose size and amino acid composition are unique to each species [4]. The structure of PVD shows great variability among the species, and even between strains of the same species. PVDs are not only iron chelators, but also act as signaling molecules for virulence, prevent iron toxicity due its overload and effectively scavenge hydroxyl and peroxyl radicals [5] [6].

The fluorescent *P. pseudomonas* spp. such as *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and *P. syringae* are well-known producers of PVDs [7]. Of them, *P. aeruginosa* (*Pa*) is the predominant producer of PVD. *Pa* is shown as an opportunistic human pathogen in patients with compromised host defense mechanisms, particularly affecting immuno-compromised patients with severe burns [8] or those with human immunodeficiency virus infection [9]. They show cosmopolitan distribution, with inhabitance in soil, water, plants and animals. Reportedly, three distinct PVD types are being produced by strains of *Pa*, *viz.*, PVD I, PVD II and PVD III—each subtype is characterized by a different peptide chain [10]. Of them, PVD I and PVD II together contribute 84% of PVDs, *i.e.*, and the remaining 16% is represented by PVD III subtype [10].

No report is available yet in literature on the inhabitance of a *Pa* strain in the rumen of Malabari goat. Malabari goat is specifically found in the Malabar region of Kerala State, India. We have very recently reported a novel yeast [11] and a bacterium [12] from this special breed with special characteristics. In the light of this background, the objectives of the present study are to: 1) isolate and characterize novel siderophore producing *Pa* strain from the rumen of Malabari goat; 2) identify and characterize the type of PVD produced by it; and 3) quantify the purified PVD.

2. Materials and Methods

2.1. Isolation

Rumen contents from both male and female Malabari goats were collected aseptically in screw-capped tubes from the slaughter house at Chelari (11.18189600 N; 75.82206300 E), Malappuram District of Kerala State, as described [13]. Briefly, 10 ml sterile double distilled water (ddH_2O) was added to 10 g sample (rumen content) and centrifuged at 800 × g for 4˚C at 5 min. The supernatant (1 ml) obtained as above was serially diluted (up to 10^{-6}) with pre-sterilized ddH_2O [11]. This diluted sample (100 µl) was spread aseptically on nutrient agar medium (pH-7.2) in petri-dishes at 37˚C for 48 h. The isolated colonies so obtained were examined for the presence of *Pseudomonas* sp. The purity of the isolate was confirmed by sub-culturing.

2.2. Morphological and Biochemical Characterization

The bacterium was identified macro-morphologically by observing the colony characteristics such as color, texture and topography of the surface and edges; and also by micro-morphological evaluation on Gram staining. Identification up to species level has been accomplished based on these morpho-cytological as well as biochem-

ical characteristics such as fermentation reactions in the medium containing various sugars (glucose, lactose, sucrose and maltose), IMViC, starch hydrolysis and nitrate reduction tests [14].

2.3. Molecular Characterization

Genetic analysis of the isolate was performed by PCR-based 16S r-DNA analysis. Forward and reverse DNA sequencing reactions of PCR amplicon were carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The gene sequence was used to carry out BLAST with the 16S r-DNA nucleotide database of *Pseudomonas* spp., the consensus sequence obtained already submitted to NCBI GenBank database. Based on maximum identity score, first 11 sequences were selected for constructing the phylogenetic tree using MEGA 4 [15].

2.4. Growth Profile

Bacterial growth was monitored in a specially designed semi-synthetic medium—designated as BUP medium—which consisted of the following ingredients (per litre): 5 g peptone, 3 g beef extract, 5 g NH_4NO_3, 4 g NH_4SO_4, 2 g K_2HPO_4, 2 g NaCl, 0.1 g $MgSO_4$, 0.5 g cysteine-HCl and 10% ground nut oil. The experiment was initiated with 1% inoculum (v/v) from an overnight stock culture. To monitor the bacterial growth, the turbidity as well as wet weight of the culture was determined regularly at 6 h interval. Turbidity measurement was carried out spectrophotometrically at λ_{600} nm and wet weight of the cell pellet was weighed out after centrifugation (8000 × g for 10 min).

The BUP medium was used for the whole study involving pigment (PVD) production and characterization, except for CAS assay.

2.5. Characterization of the Pigment

2.5.1. Chromazurol-S (CAS) Assay

CAS assay was employed for the detection of siderophore; both in liquid and on solid-agar media [16]. For the liquid CAS assay, the pure isolate was grown in minimal medium supplemented with 3.2% piperazine-N, N'-bis (2-ethanesulphonoc acid) (MM9/PIPES), 3% casamino acids and 2% glucose [16], which was incubated at 37˚C on a rotary shaker at 133 rpm for 48 h. Color change in the medium was scored visually to confirm the production of siderophore, *i.e.*, the basic blue colour would change to brown. For solid CAS assay, about 1 mg cell pellet obtained after centrifuging (8000 × g) the culture (24 h old) in liquid CAS medium was spotted on to the centre of CAS-agar plates and incubated at 37˚C for 5 d, appearance of a clear orange halo around the spotted cell mass would be an indication for the production of siderophore.

2.5.2. Spectrophotometry

In order to check whether the siderophore produced by the novel isolate was a PVD, the culture was centrifuged (8000 × g) to separate supernatant from the cell pellet. Absorption spectrum of the culture supernatant was recorded at λ_{450} to λ_{350}.

2.5.3. Thin Layer Chromatography (TLC)

Purity of the pigments was tested by TLC in several solvent systems. Of which, *n*-butanol, acetic acid and water in a ratio of 3:1:1 has been reported in this study. For the detection of fluorescence emitted by the PVD, the TLC plates were air-dried and observed under UV-light at λ354 [17].

2.5.4. Siderosomes

4 d old culture was examined under the fluorescent microscope, Ex 425-445HQ, Em 460-510HQ, Dc 450 (filter specification) for the identification of siderosomes.

2.5.5. Isoelectric Focusing (IEF)

PVDs were analyzed by IEF according to the method described earlier [7] and the visualized bands were compared with those from the representative strains of each PVD type, *viz.*, *P. aeruginosa* strain *Pa* O1 (*Pa* O1), *P. aeruginosa* strain ATCC 27853 (*Pa* ATCC 27853) and *P. aeruginosa* strain 6 (*Pa*6).

2.5.6. Pigment Purification and Quantification

After 4 d incubation, the culture in BUP medium was centrifuged (8000 × g) to separate the supernatant from the cell pellet. Subsequently, the supernatant was acidified to pH 6 using 12 M HCl; then the culture supernatant was passed through an XAD-4 Amberlite column (15 × 2.5 cm), which retained the pyoverdines [4]. After washing with 500 ml ddH_2O, the pyoverdine was eluted with 250 ml 50% methanol, the eluent was vacuum dried and lyophilized. The dried pigment was weighed for quantification.

3. Statistics

In order to check the reproducibility of the results, all studies were repeated at least thrice, and an average of 3 consistent values is presented with standard deviation. Microsoft Excel was used to draw the figures.

4. Results

4.1. Identification and Characterization of the Isolate

Based on morphological, microbiological, biochemical and molecular characteristics, the novel bacterial isolate from the rumen of Malabari goat was confirmed as *P. aeruginosa* (*Pa*), which was designated as *Pa* strain BUP2. The sequence was deposited with GenBank (NCBI GenBank Accession No. **JQ 407054**).

Upon of incubation at 37˚C for 2 d, the pure *Pa* strain BUP2 colony on nutrient-agar medium appeared smooth, round with scalloped margin on nutrient agar in petri-dishes **Figure 1(A)**. Microscopic characters were typical to Pseudomonads with rod-shaped, asporogenous, Gram-negative cells measured 0.4 to 0.8 (width) by 1 to 2 µm (length) **Figure 1(B)**. The culture was characterized biochemically by carbohydrate fermentation, nitrate reduction, starch hydrolysis and IMViC tests, of which methyl red and starch hydrolysis tests were positive (+ve), whereas others were negative (−ve) **Table 1**. Based on nucleotide homology and phylogenetic analysis, the bacterial isolate was further confirmed as a strain of *Pa.* The phylogenetic tree in comparison with related 11 strains of *Pa* was constructed **Figure 2**.

4.2. Growth Characteristics of *Pa* BUP2 in BUP Medium

Bacterial growth in BUP medium was monitored spectrometrically, the isolate showed characteristic sigmoid pattern of growth **Figure 3(A)**. The culture showed exponential growth after 30 h of incubation and reached the plateau by 36 h in the BUP medium. Wet weight of the biomass was also in line with the growth pattern mentioned in **Figure 3(B)**. The pH profile of the culture showed a characteristic increase from initial 6.7 to 7.82, followed by a decrease after 4 d of incubation; this declining pH was associated with the increased secretion of pigment in the medium **Figure 3(C)**.

4.3. Identification and Characterization of the Pigment

Identification and characterization of the pigment were done by CAS assay, UV-Vis spectrophotometry, TLC

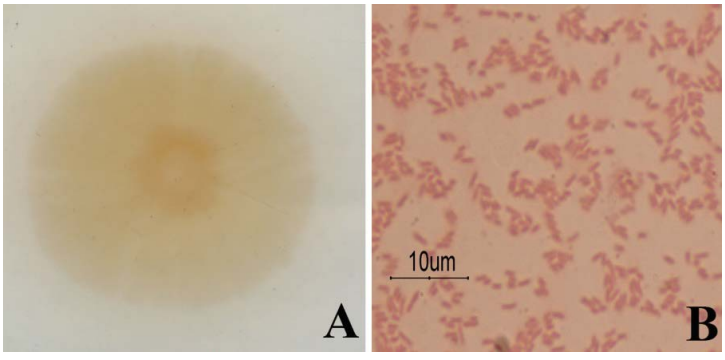

Figure 1. Morphology of *Pa* strain BUP2. (A) Digital image of the magnified view of the pure colony with smooth, round with scalloped margin on nutrient agar plates (DSLR Canon 450 D, Japan); and (B) magnified cells (100×) after Gram staining; (Image Analyzer, Nikon Eclipse E400, attached with Nikon Digital Camera DXM 1200F, Japan).

Table 1. Summary of biochemical tests performed on *P. aeruginosa* BUP2.

Biochemical Tests	Results
Citrate Utilization Test	−ve
Carbohydrate Fermentation Test	
Glucose Fermentation	−ve
Maltose Fermentation	−ve
Sucrose Fermentation	−ve
Lactose Fermentation	−ve
Nitrate Reduction Test	−ve
Indole Production Test	−ve
Voges-Proskauer Test	−ve
Starch Hydrolysis Test	+ve
Methyl Red Test	+ve

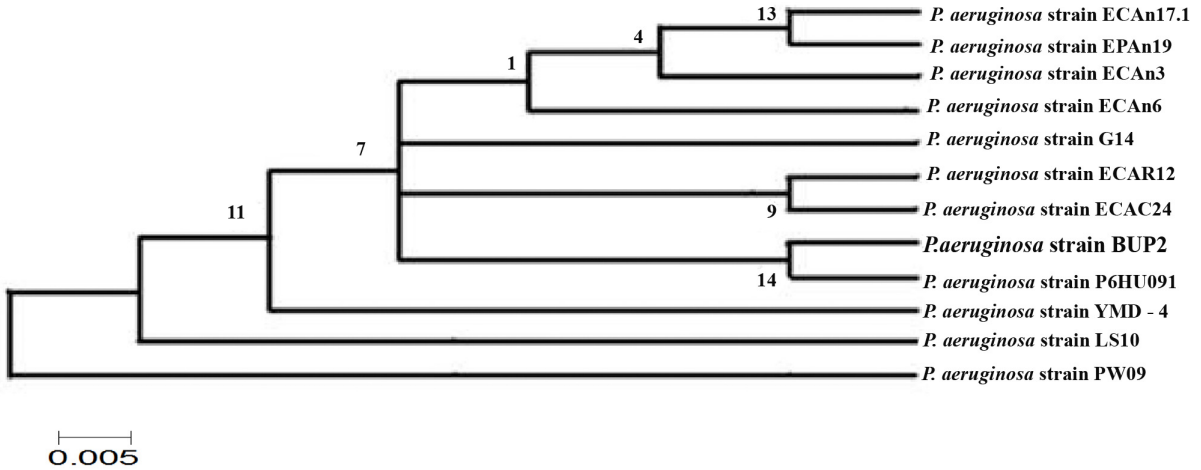

Figure 2. Evolutionary relationships of *Pa* strain BUP2 with other 11 strains of *P. aeruginosa*.

and IEF. The first objective was to identify if the pigment was a siderophore, for which CAS assay was performed. Secondly, we wanted to check whether the siderophore was a PVD, for which UV-Vis spectrophotometric and TLC assays were performed. Thirdly, we wanted to confirm whether the PVD was a new type or coming under one of the already known subtypes.

4.3.1. CAS Assay

Conventionally, CAS assay would be performed on solid agar medium in petri-dishes (solid state). In this study, we employed both liquid and solid states for CAS assay **Figures 4(A)-(C)**. Compared to solid-state, the performance was better in liquid state. The pigment was characterized by CAS assay, a universal siderophore detection method in which CAS act as an indicator. The blue color dye, CAS turns its color in to orange on removal of iron from the medium by the siderophore produced. The CAS along with HDTMA (hexadecyltrimethylammonium bromide, the detergent) has high affinity towards iron and produces a blue color. When a strong iron chelator in the medium (secreted by the growing culture) such as siderophore, removes iron from the dye complex, the color changes from blue to orange with a typical orange halo around the colonies, indicating the production of siderophores by *Pa* strain BUP2 **Figure 4(A)**. This result was also in corroboration with that in liquid CAS medium **Figure 4(B)** and **Figure 4(C)**. Thus, the pigment produced by *Pa* strain BUP2 was confirmed as a siderophore [16].

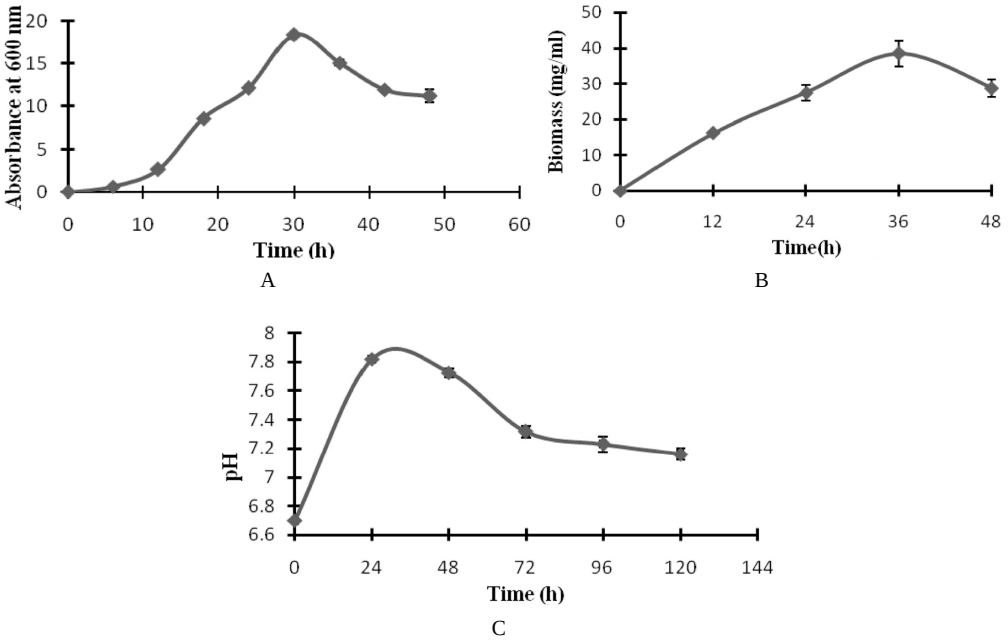

Figure 3. Growth characteristics of Pa BUP2 in BUP medium. (A) Growth curve, absorbance at λ_{600}; (B) Wet weight of the culture pellet obtained after centrifuging at 8000 × g for 10 min; and (C) pH change in culture during incubation.

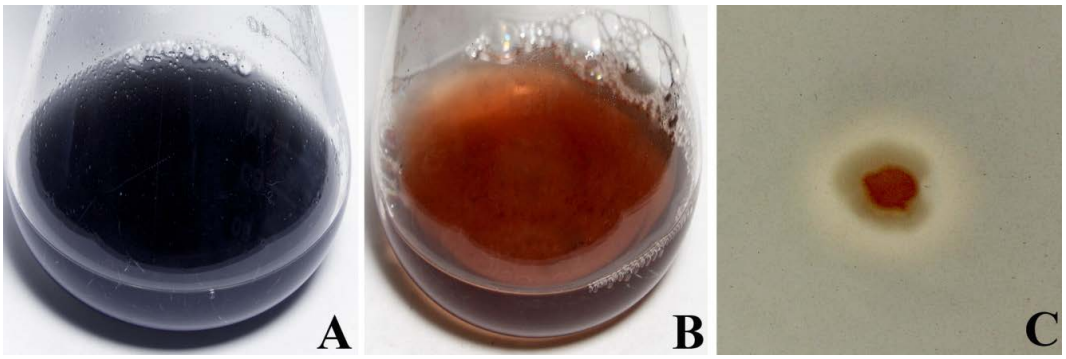

Figure 4. Characterization of the pigment by CAS assay. (A) Liquid CAS medium in blue color before inoculation; (B) Liquid CAS medium changed its color to orange after 48 h incubation indicating siderophore production; (C) CAS agar medium shows siderophore production.

4.3.2. Spectrophotometry

The preliminary indication of the possible identity of the pigment was generally provided by its absorption spectrum. Upon scanning, the absorption maximum for the pigment was obtained at λ_{404} **Figure 5**. Thus, the characteristic peak obtained at λ_{404} was of PVD [18].

4.3.3. Thin Layer Chromatography (TLC)

Identification of the siderophore type was done by TLC. Profile of extracted pigment on silica gel G250 chromatogram showed a single spot with an R_f of 0.8 on butanol: acetic acid: water (3:1:1) solvent system. As shown in **Figure 6**, the spot was visible as yellowish-brown in visible light and bright-orange upon UV irradiation (λ = 354 nm), which is the characteristic color property of PVD [17].

4.3.4. Siderosomes

Copious number of sidersomes observed as blue fluorescent granules in the terminal region of bacteral cell **Figure 7**.

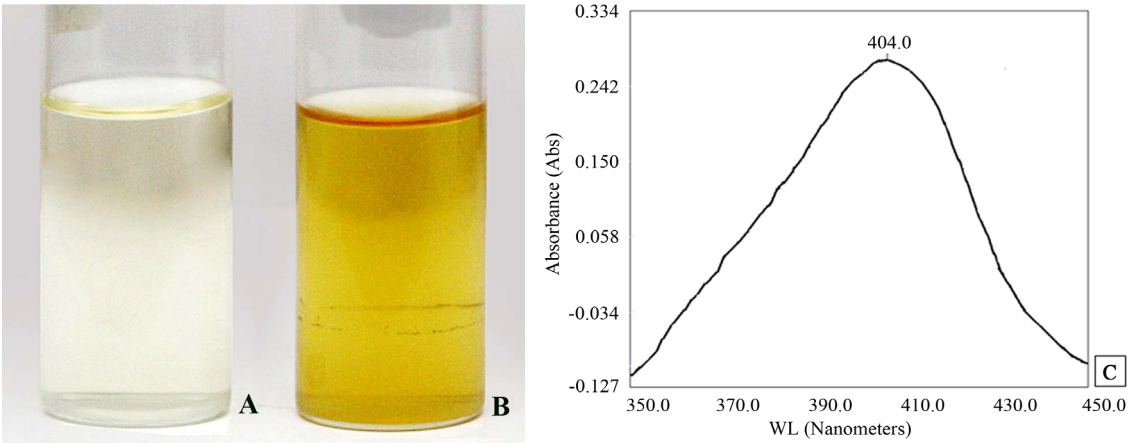

Figure 5. Characterization of the pigment. (A) Control for pigment extraction; (B) Extracted yellowish green pigment in ethyl acetate; (C) Characterization of the pigment by spectrophotometry. The culture supernatant obtained after centrifuging at 8000 × g was monitored for its absorption spectrum from λ_{350} to λ_{450}. Maximum absorption at λ_{404} indicates the pigment is PVD.

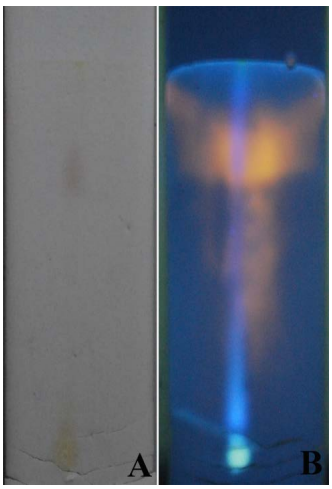

Figure 6. Identification of the type of siderophore by TLC. (A) Separation of extracted pigment on silica gel G250 chromatogram showing a single yellowish brown spot R_f of 0.8. in visible light; and (B) Purified siderophore spot visible as bright orange upon UV irradiation (354 nm), which is the characteristic color property of PVD.

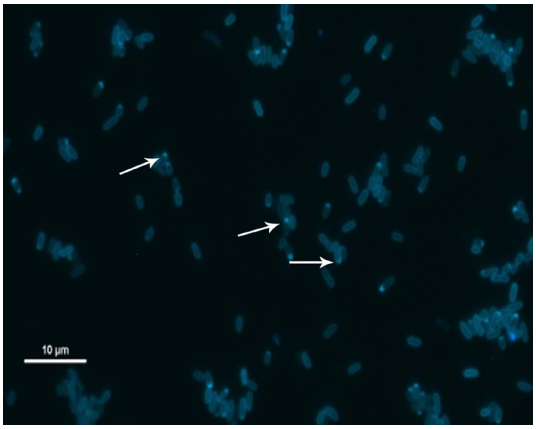

Figure 7. Identification of bacterial siderosomes by fluorescent microscope (arrows were pointed out the siderosome inside the bacterial cell).

4.3.5. Iso-Electric Focusing (IEF)

The IEF analysis was performed for judging the type of PVD produced by *Pa* strain BUP2. As depicted in **Figure 8**, we have compared the PVD produced by *Pa* strain BUP2 with that of 3 known type strains of *Pa*, *viz.*, *Pa* O1, ATCC 27853 and *Pa*6. From IEF profile, we confirmed that the PVD produced by *Pa* strain BUP2 was type 2 PVD [10].

4.3.6. Purification and Quantification of PVD

PVD production was monitored for 5 d with 24 h interval. **Figure 9** depicts the pigment production profile of *Pa*, as recorded from the absorbance at λ_{404}. Maximum pigment production was observed in the medium on 4 d of incubation with an OD of 1.945; which gradually declined to 1.888 after 5 d. It was at this higher OD (1.945), concomitant production of maximum pigment (dry weight 11.17 mg/mL) was obtained from the culture supernatant **Figure 10**.

5. Discussion

Malabari goat is a special breed of domestic mammal confined to the Malabar (Northern part) region of Kerala

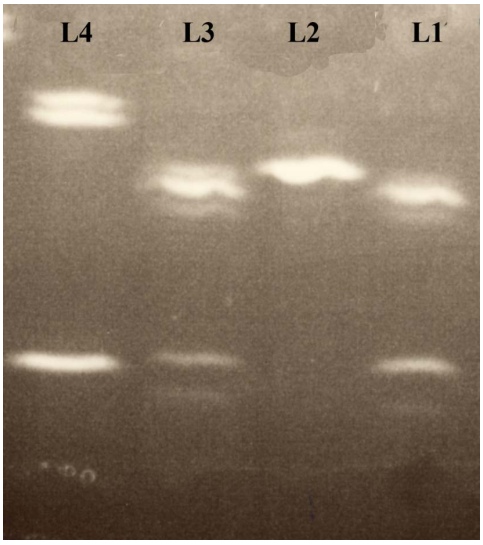

Figure 8. Characterization of PVD by IEF. PVD produced by *Pa* strain BUP2 (Lane 1) was compared with that of 3 known type specimens, *viz.*, *Pa* O1 (Lane 4, type 1), *Pa* ATCC 27853 (Lane 3, type 2) and *Pa*6 (Lane 2, type 3), indicating its close similarity to type 2.

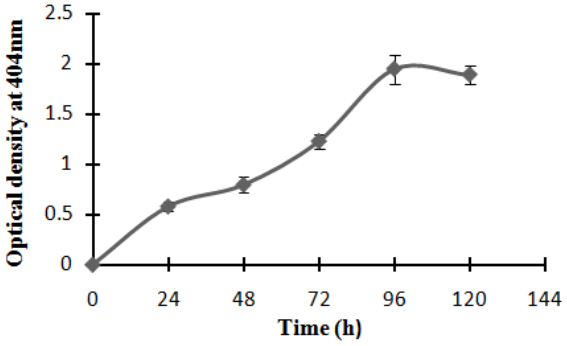

Figure 9. Pigment production profile of the *Pa* BUP2. At 24 h intervals, the culture supernatant was monitored for PVD production at λ_{404}. Maximum PVD production was observed in the BUP medium at 4th d incubation (OD 1.945), which gradually declined to 1.888 after 5th d of incubation.

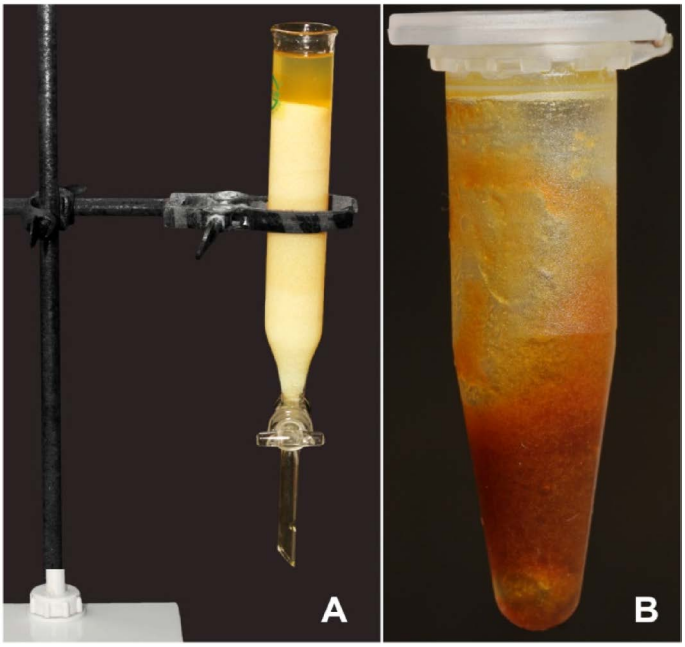

Figure 10. Pigment purification and quantification**.** (A) Acidified supernatant were loaded with XAD-4 Amberlite column; (B) Purified and dried pyoverdine in Eppendorf tube.

state, India. We explored for the microflora inhabiting the rumen of this goat [19]. Very recently, we reported an efficient yeast capable of producing biosurfactant and poly hydroxy butyrate isolated from rumen content [11] and a bacterium producing lipase [12]. Along with this, we have isolated *P. aeruginosa* producing large quantity of PVD type 2, whose isolation and purification are reported herein.

P. aeruginosa is a Gram-negative, rod-shaped and asporogenous bacterium isolated from various clinical samples [20]. *Pa* inhabits in soil, water, skin flora, and most of the man-made environments throughout the world. Due to its ubiquitous inhabitance in the environment, many animals transiently harbor this bacterium. Apart from humans, various reports are available in literature regarding the inhabitance of *Pa* in the rumen, intestine, milk and fecal matter of cattle and calves including sheep, camel, etc. [21]-[23]. As far as literature says, this is the first report on the inhabitance and characterization of a *Pa* strain from the rumen of a goat, especially Malabari goat. In fact, this bacterium was initially a facultative anaerobe, which we tuned to be an aerobe by growing in a specially designed flask, the Benjamin flask [11].

Pa is often observed as "growing in distilled water" bacterium, an evidence of its minimal nutritional requirements [24]. *Psuedomonas* spp. were generally cultured in King B medium [6] and various other synthetic media [25] [26] for growth and pigment production. In this study, we designed a new medium (BUP medium) that made the culture well adapted for growth and pigment production, even after drastic change of its ecological niche from the rumen environment (anaerobic state) to artificial liquid conditions (aerobic state). *Pa* strain BUP2 showed characteristic sigmoid growth curve in BUP medium. *Pa* BUP2 secreted a yellow-green pigment in BUP medium during its late senescence phase (96 h) of growth, which made the culture clear and transparent. The pH profile of the culture showed a characteristic increase 6.7 to 7.9 initially, followed by a decrease after 4 d of incubation, this may be due the slightly alkaline nature of the pigment as described [18].

The major objective of this study was to isolate and characterize the pigment qualitatively and quantitatively at regular intervals during its growth. Having confirmed the isolate as a novel strain of *Pa*, we proceeded further to confirm if the pigment produced was a siderophore. Strains of *Pa* are known to produce a variety of pigments such as pyorubrin, pyomelanin, pyocyanin, pyoverdines, etc., with color variations from blue to brown [27] Upon CAS assay, *Pa* strain BUP2 showed a vivid positive result for siderophore production. Removal of iron from CAS/HDTMA complex medium by siderophores turned its color from blue to brown/orange. The orange halo around the colony [16] further confirmed the production of siderophores. Only a few studies to date, reported the use of liquid medium for CAS assay [28]. We used both liquid and solid media to confirm the

pigment produced as a siderophore.

Secondly, we wanted to confirm if the siderophore produced was a PVD. *Pa* possesses several virulence factors, one of which is PVD, a yellow-green primary siderophore that sequesters iron from the host to facilitate its growth, thereby causing iron depletion in the host as reflected through various diseases [29]. The absorption spectrum of the extracted siderophore showed a characterestic peak of PVD with absorption maximum at 404 nm. According to [18] and [30], peak at λ_{404} is of a typical PVD. TLC was used to check the purity of the sample. Single spot obtained on TLC with orange fluorescence upon UV irradiation further illustrated the production of PVD [17].

Thirdly, we wanted to identify the type of PVD produced by *Pa* strain BUP2. Siderotyping is the most efficient and effective method for the characterization of novel PVDs. Strains producing structurally different PVDs are immediately distinguished from each other from typical PVD-IEF patterns. Three distinct PVD types are known to produce by the strains of *Pa*, *viz.*, PVD I, PVD II and PVD III that are distinguishable from their peptides at molecular level and IEF patterns [10]. Generally, PVDs of strains 9 AW, *Pa* 15692, 62.5, 62.6, 71.20, 77.10 and 9 BW are included in group I, PVDs of strains *Pa* 27853, 63.52, 72.26, 76.110, l W, 1 OCW and *P. fluorescens* ATCC 13525 in group II, and the group III is for the PVDs of strain 51 W and 77.11; all these subtypes can clearly be distinguished by IEF [10]. We compared the PVD produced by *Pa* strain BUP2 with that of three type *Pa* strains, *i.e.*, *Pa* O1, ATCC 27853 and *Pa*6, the representatives of type 1, type 2 and type 3, respectively. The pattern of bands obtained on IEF for *Pa* strain BUP2 clearly corroborates with that of PVD II [7]. Thus, we concluded that the PVD produced by *Pa* BUP2 belongs to type 2 PVD.

Our next objective was to quantify the pigment gravimetrically. The column fraction (XAD-4 Amberlite) of extracted pigment was evaporated to dryness and weighed [4]. Thus, we quantified pure PVD type 2 produced by *Pa* strain BUP2.

6. Conclusion

In conclusion, no *Pa* strain has been characterized from the rumen of a goat, as reported in this study. Briefly, we confirmed that the the bacterium isolated from the rumen content of Malabari goat is a novel strain of *Pa* capable of producing copious quantity of PVD II in specially designed BUP medium under aerobic conditions. This study looks forward to elaborating studies with *Pa* strain BUP2 to elucidate its clinical and industrial significances.

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